



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hu *et al.*

Serial No.: 09/854,844

Group Art Unit: 1652

Filed: 05/14/2001

Examiner: D. Ramirez

For: Novel Human Protease and Polynucleotides Attorney Docket No.: LEX-0176-USA
Encoding the Same

RECEIVED
JUL 10 2003
TECH CENTER 1600/2900

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Assistant Commissioner for Patents
Alexandria, VA 22313

BEST AVAILABLE COPY



24231

PATENT TRADEMARK OFFICE



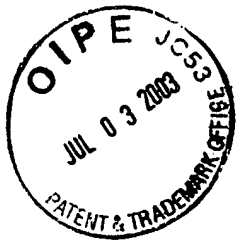
TABLE OF CONTENTS

I.	REAL PARTY IN INTEREST	1
II.	RELATED APPEALS AND INTERFERENCES	1
III.	STATUS OF THE CLAIMS	2-3
IV.	STATUS OF THE AMENDMENTS	3
V.	SUMMARY OF THE INVENTION	3-4
VI.	ISSUES ON APPEAL	4
VII.	GROUPING OF THE CLAIMS	4
VIII.	ARGUMENT	4-25
A.	Do Claims 1-8 Lack a Patentable Utility?	4-17
B.	Are Claims 1-8 Unusable Due to a Lack of Patentable Utility?	17-18
C.	Is Claim 2 Indefinite?	18-19
D.	Do Claims 1, 5 and 8 Meet The Written Description Requirement?	19-22
E.	Are Claims 1, 5 and 8 Enabled?	22-25
IX.	APPENDIX	26
X.	CONCLUSION	27

RECEIVED

JUL 10 2003

TECH CENTER 1600/2900



APPEAL BRIEF

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences ("the Board") in response to the Final Office Action mailed on December 2, 2002. The Notice of Appeal was timely submitted on March 31, 2003, and was received in the Patent and Trademark Office ("the Office") on April 3, 2003. This Appeal Brief is timely submitted in light of the concurrently filed Petition for an Extension of Time of one month to and including July 3, 2003, and authorization to deduct the fee as required under 37 C.F.R. § 1.17(a)(1) from Appellants' Representatives' deposit account. The Commissioner is also authorized to charge the fee for filing this Appeal Brief (\$160.00), as required under 37 C.F.R. § 1.17(c), to Lexicon Genetics Incorporated Deposit Account No. 50-0892.

Appellants believe no fees in addition to the fee for filing the Appeal Brief and the fee for the extension of time are due in connection with this Appeal Brief. However, should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason related to this communication, the Commissioner is authorized to charge any underpayment or credit any overpayment to Lexicon Genetics Incorporated Deposit Account No. 50-0892.

I. REAL PARTY IN INTEREST

The real party in interest is the Assignee, Lexicon Genetics Incorporated, 8800 Technology Forest Place, The Woodlands, Texas, 77381.

II. RELATED APPEALS AND INTERFERENCES

Appellants know of no related appeals or interferences that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

RECEIVED
JUL 10 2003
TECH CENTER 1600/2900

III. STATUS OF THE CLAIMS

The present application was filed on May 14, 2001, claiming the benefit of U.S. Provisional Application Number 60/205,275, which was filed on May 18, 2000, and included original claims 1-4. A First Official Action on the merits ("the First Action") was issued on December 3, 2001, in which claims 1-3 were rejected under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1-4 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, claims 1-3 were variously rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite, and claim 1 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking sufficient written description. In a response to the First Action submitted to the Office on March 1, 2002 ("Response to the First Action"), Appellants amended claims 1-3 to further improve their clarity and addressed the rejections of claims 1-4.

A Second and Non-Final Official Action on the merits ("the Second Action") was issued on May 7, 2002, indicating that the rejection of claims 1 and 3 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite had been overcome by the amendments and remarks submitted in the Response to the First Action, but maintaining the rejection of claims 1-4 under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1-4 under 35 U.S.C. § 112, first paragraph, as allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, claim 2 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite, and claim 1 under 35 U.S.C. § 112, first paragraph, as allegedly lacking sufficient written description, and newly rejecting claim 1 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. In a response to the Second Action submitted to the Office on September 5, 2002 ("Response to the Second Action"), Appellants added claims 5-8, and addressed the rejections of claims 1-4.

A Third and Final Official Action ("the Final Action") was mailed on December 2, 2002, maintaining the rejection of claims 1-4 (and including newly added claims 5-8) under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1-4 (and including newly added claims 5-8) under 35 U.S.C. § 112, first paragraph, as allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, claim 2 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite, claim 1 (and including newly added claims 5 and 8) under 35 U.S.C. § 112, first paragraph, as allegedly lacking

sufficient written description, and claim 1 (and including newly added claims 5 and 8) under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. In a response to the Final Action submitted on March 31, 2003 ("Response to the Final Action"), Appellants again addressed the rejections of claims 1-8. An Advisory Action ("the Advisory Action") was mailed on April 29, 2003, maintaining the rejection of claims 1-8 under 35 U.S.C. § 101 as allegedly lacking a patentable utility, claims 1-8 under 35 U.S.C. § 112, first paragraph, as allegedly unusable by the skilled artisan due to the alleged lack of patentable utility, claim 2 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite, claims 1, 5, and 8 under 35 U.S.C. § 112, first paragraph, as allegedly lacking sufficient written description, and claims 1, 5, and 8 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. Therefore, claims 1-8 are the subject of this appeal. A copy of the appealed claims are included below in the Appendix (Section IX).

IV. STATUS OF THE AMENDMENTS

As no amendments subsequent to the Final Action have been filed, Appellants believe that no outstanding amendments exist.

V. SUMMARY OF THE INVENTION

The present invention relates to Appellants' discovery and identification of novel human polynucleotide sequences that encode a novel protein that shares structural similarity with mammalian proteases (specification at page 1, lines 11-12), and particularly serine proteases (specification at page 2, lines 1-2).

The presently claimed polynucleotide sequences were compiled from gene trapped cDNAs, in conjunction with cDNAs prepared from human brain, cerebellum, testis, kidney, skeletal muscle, thymus and salivary gland mRNAs (specification at page 3, lines 11-13). Two coding single nucleotide polymorphisms were identified in the claimed sequence - specifically, a G/A polymorphism at nucleotide position 343 of SEQ ID NO:1, which can result in a valine or isoleucine being present at corresponding amino acid position 115 of SEQ ID NO:2; and a C/T polymorphism at nucleotide position 868 of

SEQ ID NO:1, which can result in a cysteine or arginine being present at corresponding amino acid position 290 of SEQ ID NO:2.

The specification details a number of uses for the presently claimed polynucleotide sequences, including in diagnostic assays such as forensic analysis (see, for example, the specification at page 10, line 28), in identification of protein coding sequence and identification of exon splice junctions (see, for example, the specification at page 2, lines 23-25, and page 10, lines 28-33), in mapping the sequences to a specific region of a human chromosome (see, for example, the specification at page 2, lines 25-26), and in assessing gene expression patterns, particularly using a high throughput “chip” format (see, for example, the specification at page 5, lines 18-21).

VI. ISSUES ON APPEAL

1. Do claims 1-8 lack a patentable utility?
2. Are claims 1-8 unusable by a skilled artisan due to a lack of patentable utility?
3. Is claim 2 indefinite?
4. Do claims 1, 5 and 8 lack sufficient written description?
5. Are claims 1, 5 and 8 enabled?

VII. GROUPING OF THE CLAIMS

For the purposes of the outstanding rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, associated with the utility rejection, the claims will stand or fall together. For the purposes of the outstanding rejection under 35 U.S.C. § 112, second paragraph, claim 2 will stand or fall alone. For the purposes of the outstanding rejections under 35 U.S.C. § 112, first paragraph, associated with written description and enablement, claims 1, 5, and 8 will stand or fall together.

VIII. ARGUMENT

A. Do Claims 1-8 Lack a Patentable Utility?

The Final Action first rejects claims 1-8 under 35 U.S.C. § 101, as allegedly lacking a patentable

utility due to not being supported by either a specific and substantial or a well-established utility.

Appellants pointed out in the Response to the Final Action that the present nucleic acid sequences have utility in forensic analysis, as described in the specification as originally filed (see, for example, page 10, line 28). As described in the specification from page 15, line 29 to page 16, line 2, the presently claimed sequence defines two coding single nucleotide polymorphisms - specifically, a G/A polymorphism at nucleotide position 343 of SEQ ID NO:1, which can result in a valine or isoleucine being present at corresponding amino acid position 115 of SEQ ID NO:2; and a C/T polymorphism at nucleotide position 868 of SEQ ID NO:1, which can result in a cysteine or arginine being present at corresponding amino acid position 290 of SEQ ID NO:2. As such polymorphisms are the basis for forensic analysis, which in undoubtedly a “real world” utility, the presently claimed sequence must in itself be useful.

The Advisory Action states that the use of the present sequences in forensic analysis is not a specific utility because “it is unclear to the Examiner as to how this can be a specific utility for the claimed polynucleotides absent an indication as to how these polymorphisms can be used to distinguish between one person from another” (the Advisory Action at page 3). Appellants respectfully point out that the presently described polymorphisms can be used by those skilled in the art to “distinguish between one person from another” simply based on the presence or absence of the described polymorphism. The Examiner has provided no evidence of record that establishes that skilled artisans would not be able to use the presently described polymorphisms in forensic analysis exactly as they were described in the specification as originally filed, without any additional research. It is important to note that simply because the use of these polymorphic markers will necessarily provide additional information on the percentage of particular subpopulations that contain these polymorphic markers does not mean that additional research is needed in order for these markers as they are presently described in the instant specification to be used in forensic science. Thus, the Examiner has failed to meet her evidentiary burden of proving that the present invention lacks utility.

This is also not a case of a potential utility. In the response to the Final Action, Appellants pointed out that even in the worst case scenario, the described polymorphisms are each useful to distinguish 50% of the population (in other words, the marker being present in half of the population). The Advisory Action

states that “the Examiner has not been able to locate any support in the specification in regard to this assertion nor can the Examiner find any information in the art in regard to how one can use these specific polymorphisms in the claimed polynucleotides as a marker to distinguish 50% of the population” (the Advisory Action at page 3). First, Appellants point out that the ability of a polymorphic marker to distinguish at least 50% of the population is an inherent feature of any polymorphic marker, and this feature is well understood by those of skill in the art. Appellants note that as a matter of law, it is well settled that a patent need not disclose what is well known in the art. *In re Wands*, 8 USPQ 2d 1400 (Fed. Cir. 1988). Second, the assertion that the present claims lack utility because the Examiner cannot “find any information in the art in regard to how one can use these specific polymorphisms in the claimed polynucleotides as a marker to distinguish 50% of the population” (the Advisory Action at page 3, emphasis added) strains credulity. The Examiner seems to be suggesting that because the presently claimed sequences and polymorphisms are novel, that they cannot have a patentable utility. However, this is clearly not the standard under 35 U.S.C. § 101. Appellants respectfully point out that all that is required to support Appellants assertion of utility is for the skilled artisan to believe that the presently described polymorphic markers could be useful in forensic analysis. The fact that forensic biologists use polymorphic markers such as those described by Appellants every day provides more than ample support for the assertion that forensic biologists would also be able to use the specific polymorphic markers described by Appellants in the same fashion. Therefore, these allegations are completely without merit, and in no way establish that the present invention lacks utility.

Additionally, the Examiner seems to be confusing the requirements of a specific utility with a unique utility. The fact that other polymorphic markers have been identified in other genetic loci, or that the use of the presently described polymorphic markers will provide additional information concerning the prevalence of these markers in certain subpopulations, does not mean that Appellants’ identification of polymorphic markers in SEQ ID NO:1 is not specific. As clearly stated by the Federal Circuit in *Carl Zeiss Stiftung v. Renishaw PLC*, 20 USPQ2d 1101 (Fed. Cir. 1991):

An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: “[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding a

lack of utility.” *Envirotech Corp. v. Al George, Inc.*, 221 USPQ 473, 480 (Fed. Cir. 1984)

In other words, just because other (possibly better) polymorphic markers from the human genome have been described, or that additional information about the presently described polymorphic markers can be gained through the use of these markers, does not establish that the presently described polymorphic markers lack a specific utility. The requirement for a specific utility, which is part of the standard for utility under 35 U.S.C. § 101 presently being applied by the Office, should not be confused with the requirement for a unique utility, which is not the legal standard. If every invention were required to have a unique utility, the Patent and Trademark Office would no longer be issuing patents on batteries, automobile tires, golf balls, golf clubs, and treatments for a variety of human diseases, just to name a few particular examples, because other examples of each of these have already been described and patented. However, only the briefest perusal of virtually any issue of the Official Gazette provides numerous examples of patents being granted on each of the above compositions every week. Furthermore, if each invention needed to have a unique utility in order to be patented, the entire class and subclass system would be an effort in futility, as the class and subclass system serves solely to group such common inventions, which would not be required if each invention needed to have a unique utility. In view of the above standards and “common sense” analysis, there can be little question that the present sequence clearly meets the requirements of 35 U.S.C. § 101.

Furthermore, as the presently described polymorphisms are a part of the family of polymorphisms that have a well established utility, the Federal Circuit’s holding in *In re Brana*, (34 USPQ2d 1436 (Fed. Cir. 1995), “*Brana*”) is directly on point. In *Brana*, the Federal Circuit admonished the Patent and Trademark Office for confusing “the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption”. *Brana* at 1442. The Federal Circuit went on to state:

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant provide regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago.

Brana at 1439, emphasis added. The choice of the phrase “utility or usefulness” in the foregoing quotation is highly pertinent. The Federal Circuit is evidently using “utility” to refer to rejections under 35 U.S.C. § 101, and is using “usefulness” to refer to rejections under 35 U.S.C. § 112, first paragraph. This is made evident in the continuing text in *Brana*, which explains the correlation between 35 U.S.C. §§ 101 and 112, first paragraph. The Federal Circuit concluded:

FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

Brana at 1442-1443, citations omitted, emphasis added. As set forth above, the present polymorphisms are useful in forensic analysis as described in the specification as originally filed, without the need for any further research. As discussed above, even if the use of these polymorphic markers provided additional information on the percentage of particular subpopulations that contain these polymorphic markers, this would not mean that “additional research” is needed in order for these markers as they are presently described in the instant specification to be of use to forensic science. As stated above, using the polymorphic marker as described in the specification as originally filed can definitely distinguish members of a population from one another. However, even if, *arguendo*, further research might be required in certain aspects of the present invention, this does not preclude a finding that the invention has utility, as set forth by the Federal Circuit’s holding in *Brana*, which clearly states, as highlighted in the quote above, that “pharmaceutical inventions, necessarily includes the expectation of further research and development” (*Brana* at 1442-1443, emphasis added). In assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is “undue”, not “experimentation”. *In re Angstadt and Griffin*, 190 USPQ 214 (CCPA 1976). The need for some experimentation does not render the claimed invention unpatentable. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Angstadt*

and *Griffin, supra*; *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991). Again, as a matter of law, it is well settled that a patent need not disclose what is well known in the art (*In re Wands, supra*).

Although Appellants need only make one credible assertion of utility to meet the requirements of 35 U.S.C. § 101 (*Raytheon v. Roper*, 220 USPQ 592 (Fed. Cir. 1983); *In re Gottlieb*, 140 USPQ 665 (CCPA 1964); *In re Malachowski*, 189 USPQ 432 (CCPA 1976); *Hoffman v. Klaus*, 9 USPQ2d 1657 (Bd. Pat. App. & Inter. 1988)), Appellants noted in the response to the First Action, the response to the Second Action, and the response to the Final Action, as a further example of the utility of the presently claimed polynucleotide, as described in the specification at least at page 2, lines 24-26, the present nucleotide sequences have a specific utility in “identification of protein coding sequence” and “mapping a unique gene to a particular chromosome”. This is evidenced by the fact that SEQ ID NO:1 can be used to map the 5 coding exons of the gene comprising the presently claimed sequence on chromosome 4 (present within a chromosome 4 clone; Genbank Accession Number AC104819; alignment and the first page from the Genbank report are presented in **Exhibit A**). Appellants respectfully remind the Board that only a minor percentage (2-4%) of the genome actually encodes exons, which in-turn encode amino acid sequences. The presently claimed polynucleotide sequence provides biologically validated empirical data (*e.g.*, showing which sequences are transcribed, spliced, and polyadenylated) that *specifically* define that portion of the corresponding genomic locus that actually encodes exon sequence. Equally significant is that the claimed polynucleotide sequence defines how the encoded exons are actually spliced together to produce an active transcript (*i.e.*, the described sequences are useful for functionally defining exon splice-junctions). Such biologically validated splice junctions are superior to splice junctions that may have been predicted from genomic sequence alone, and, as detailed in the specification, at least at page 10, lines 28-33, that “sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (*e.g.*, splice acceptor and/or donor sites), *etc.*, that can be used in diagnostics and pharmacogenomics”. Appellants respectfully submit that the practical scientific value of biologically validated, expressed, spliced, and polyadenylated mRNA sequences is readily apparent to those skilled

in the relevant biological and biochemical arts.

Clearly, the present polynucleotide provides exquisite specificity in localizing the specific region of human chromosome 4 that contains the gene encoding the given polynucleotide, a utility not shared by virtually any other nucleic acid sequences. In fact, it is this specificity that makes this particular sequence so useful. Early gene mapping techniques relied on methods such as Giemsa staining to identify regions of chromosomes. However, such techniques produced genetic maps with a resolution of only 5 to 10 megabases, far too low to be of much help in identifying specific genes involved in disease. The skilled artisan readily appreciates the significant benefit afforded by markers that map a specific locus of the human genome, such as the present nucleic acid sequence. For further evidence in support of the Appellants' position, the Board is requested to review, for example, section 3 of Venter *et al.* (2001, Science 291:1304, at pp. 1317-1321, including Fig. 11 at pp.1324-1325; **Exhibit B**), which demonstrates the significance of expressed sequence information in the structural analysis of genomic data. The presently claimed polynucleotide sequence defines a biologically validated sequence that provides a unique and specific resource for mapping the genome essentially as described in the Venter *et al.* article. Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101.

The Examiner also questions these asserted utilities in the Advisory Action. The Examiner states that "while Applicants assert that the claimed polynucleotide encodes 5 exons, no empirical determination has been made to corroborate that the claimed polynucleotide contains 5 exons" (The Advisory Action bridging pages 5 and 6). Appellants are completely at a loss to understand what corroboration is required to confirm that, as asserted by Appellants, and shown above in **Exhibit A**, that the presently claimed sequence contains 5 exons. In **Exhibit A**, Appellants have conducted the exact analysis used by those of skill in the art to determine the number of exons present in a cDNA sequence - specifically, by comparing the cDNA sequence (in this case, SEQ ID NO:1) to human genomic sequence, the exons present in the cDNA clone are indicated by stretches of homologous sequences in one or more genomic clones that are separated by introns (which, by definition, are missing from the cDNA clone). The Advisory Action further states that "it is unclear to the Examiner as to how the information provided by Applicants is validated empirical data or if one can use the claimed polynucleotide to map coding exons" (the Advisory Action at

page 6, emphasis added). By providing the information shown in **Exhibit A**, there can be no doubt that the presently claimed sequence can be used to “map coding exons”. Thus, the Examiner’s position does not support the alleged lack of utility.

With regard to the utility of mapping the protein coding regions of chromosome 4, the Advisory Action states that this utility is not specific because “any polynucleotide in human chromosome 4 can be used to identify that chromosome” (the Advisory Action at page 5). This argument fails to support the Examiner’s allegation of a lack of utility in at least two respects. First, Appellants respectfully point out that while non-coding nucleotide sequences from this precise region of chromosome 4 could be used to map the introns and exons as described above, it would only be possible using the information provided by Appellants in the specification as originally filed - specifically, one needs to know which sequences correspond to the coding regions in order to use non-coding sequences to map intron/exon junctions. This is a classic case of hindsight reconstruction, using the information provided for the first time in Appellants own application against them in an attempt to question Appellants assertion of utility, and does not serve as a proper foundation for such an allegation. Second, and most importantly, the Examiner again seems to be confusing the requirements of a specific utility with a unique utility. The fact that a small number of other nucleotide sequences could be used to map the protein coding regions in this specific region of chromosome 4 does not mean that the use of Appellants’ sequence to map the protein coding regions of chromosome 4 is not specific (*Carl Zeiss Stiftung v. Renishaw PLC, supra*).

Additionally, Appellants noted in the response to the First Action, the response to the Second Action, and the response to the Final Action, that a sequence sharing 99% percent homology over an extended region with the described sequence is present in the leading scientific repository for biological sequence data (GENBANK), and has been annotated by third party scientists *wholly unaffiliated with Appellants* a sequence “similar to epidermis specific serine protease” from humans (GenBank accession number XM_093852; alignment shown in **Exhibit C**). Furthermore, there is another sequence that shares over 99% percent homology over an extended region with the described sequence, which is present in the GenBank patent database, and has been annotated by third party scientists *wholly unaffiliated with Appellants* as a “protease” (GenBank patent database accession number AX360076; alignment and patent

information provided in **Exhibit D**). The legal test for utility simply involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be credible or believable. Given these GenBank citations, there can be no doubt that those skilled in the art would clearly believe that Appellants' sequence is a serine protease.

The Examiner has repeatedly questioned Appellants' assertion that the presently claimed sequence encodes a serine protease. In the First Action, the Examiner cited Bork (Genome Research 10:398-400, 2000) as supporting the proposition that those skilled in the art would not believe that a protein has a given biochemical activity if it displays less than 70% homology with related proteins annotated as having that activity. First, even if, *arguendo*, one accepts the Examiner's contention that greater than 70% homology is required for one skilled in the art to believe that a sequence encodes a certain activity, the presently claimed sequence has exceeded the Examiner's, albeit arbitrary, threshold of sequence relatedness. Second, and more importantly, the 70% figure cited from the Bork article relates to the 70% accuracy of the resulting prediction, not 70% homology. In fact, nowhere in Bork is there a comparison of the prediction accuracy based on the percentage homology between two proteins or two classes of proteins, and thus does not support the alleged lack of utility for the present invention.

The First Action next cited Smith and Zhang (Nature Biotechnology 15:1222-1223, 1997) as teaching "that there are numerous cases in which proteins of very different functions are homologous" (the First Action at page 4). However, the Smith and Zhang article also states "the major problems associated with nearly all of the current automated annotation approaches are - paradoxically - minor database annotation inconsistencies (and a few outright errors)" (page 1222, second column, first paragraph, emphasis added). Thus, Smith and Zhang do not in fact seem to stand for the proposition that prediction of function based on homology is fraught with uncertainty, and thus also does not support the alleged lack of utility.

The First Action next cited Brenner (TIG 15:132-133, 1999) as teaching that "most homologs must have different molecular and cellular functions" (the First Action at page 4). However, this statement is based on the assumption that "if there are only 1000 superfamilies in nature, then most homologs must have different molecular and cellular functions" (page 132, second column). Furthermore, Brenner suggests that

one of the main problems in using homology to predict function is “an issue solvable by appropriate use of modern and accurate sequence comparison procedures” (page 132, second column), and in fact references an article by Altschul *et al.*, which is the basis for one of the “modern and accurate sequence comparison procedures” used by Appellants. Thus, the Brenner article also does not support the alleged lack of utility.

Finally, the First Action cited Broun *et al.* (Science 282:1315-1317, 1998) and Van de Loo *et al.* (Proc. Natl. Acad. Sci. USA 92:6743-6747, 1995) as teaching that prediction of function based on homology is unpredictable. The Final Action and the Advisory Action reiterate these citations. However, these papers cite only one example, microsomal oleate desaturase/oleate 12-hydroxylase, where function based on sequence homology proved to be incorrect. One example out of the **thousands** of predictions of function based on homology that exist in the art is hardly indicative of a high level of uncertainty, and thus also does not support the alleged lack of utility.

The Advisory Action, continuing on this theme, now cites articles by Seffernick *et al.* (J. Bacteriol. 183:2405-2410, 2001) and Witkowski *et al.* (Biochemistry 38:11643-11650, 1999) to again attempt to support the proposition that prediction of protein function from homology information is somewhat unpredictable. However, while Appellants have provided evidence of record that conclusively establishes that those skilled in the art would believe that the specifically claimed sequence encodes a serine protease, the Examiner has provided no evidence that directly establishes that the specifically claimed sequence does not encode a serine protease. Accordingly, the evidence of record compels a finding that the present invention has a patentable utility.

Furthermore, with regard to the citation of journal articles to support an allegation of a lack of utility, the PTO has repeatedly attempted to deny the utility of nucleic acid sequences based on a small number of publications that call into doubt prediction of protein function from homology information and the usefulness of bioinformatic predictions, of which these articles are merely the latest examples. Appellants readily agree that there is not 100% consensus within the scientific community regarding prediction of protein function from homology information, and further agree that prediction of protein function from homology information is not 100% accurate. However, Appellants respectfully point out that the lack of 100% consensus on prediction of protein function from homology information is **completely irrelevant**

to the question of whether the claimed nucleic acid sequence has a substantial and specific utility, and that 100% accuracy of prediction of protein function from homology information is **not the standard** for patentability under 35 U.S.C. § 101. Appellants respectfully point out that, as discussed above, the legal test for utility simply involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be **believable**. Appellants submit that the **overwhelming majority** of those of skill in the relevant art would **believe** prediction of protein function from homology information and the usefulness of bioinformatic predictions to be powerful and useful tools, as evidenced by hundreds if not **thousands** of journal articles (which Appellants will submit to the Office if the Board truly doubts Appellants' assertion that the overwhelming majority of those of skill in the art place a high value on prediction of protein function from homology information and the usefulness of bioinformatic predictions), and would thus **believe** that Appellants sequence is a serine protease. As **believability** is the standard for meeting the utility requirement of 35 U.S.C. § 101, and **not** 100% consensus or 100% accuracy, Appellants submit that the present claims must **clearly** meet the requirements of 35 U.S.C. § 101.

Thus, those of skill in the art would readily appreciate the importance of tracking the expression of the gene encoding the described protein, for example using high-throughput DNA chips, as the specification details on page 5, lines 19-21. Such "DNA chips" clearly have utility, as evidenced by hundreds of issued U.S. Patents, as exemplified by U.S. Patent Nos. 5,445,934 (**Exhibit E**), 5,556,752 (**Exhibit F**), 5,744,305 (**Exhibit G**), 5,837,832 (**Exhibit H**), 6,156,501 (**Exhibit I**) and 6,261,776 (**Exhibit J**). Evidence of the "real world" **substantial** utility of the present invention is further provided by the fact that there is an entire industry established based on the use of gene sequences or fragments thereof in a gene chip format. Perhaps the most notable gene chip company is Affymetrix. However, there are many companies that have, at one time or another, concentrated on the use of gene sequences or fragments, in gene chip and non-gene chip formats, for example: Gene Logic, ABI-Perkin-Elmer, HySeq and Incyte. In addition, one such company (Rosetta Inpharmatics) was viewed to have such "real world" value that it was acquired by large a pharmaceutical company (Merck) for significant sums of money (net equity value of the transaction was \$620 million). The "real world" **substantial** industrial utility of gene sequences or fragments would, therefore, appear to be widespread and well established. Clearly, there can be no doubt

that the skilled artisan would know how to use the presently claimed sequences (see Section VIII(B), below), strongly arguing that the claimed sequences have utility. Given the widespread utility of such "gene chip" methods using *public domain* gene sequence information, there can be little doubt that the use of the presently described *novel* sequences would have great utility in such DNA chip applications. As the present sequences are specific markers of the human genome (see above), and such specific markers are targets for the discovery of drugs that are associated with human disease, those of skill in the art would instantly recognize that the present nucleotide sequences would be ideal, novel candidates for assessing gene expression using such DNA chips. Clearly, compositions that enhance the utility of such DNA chips, such as the presently claimed nucleotide sequences, must in themselves be useful. Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101.

The Advisory Action also questions this utility, stating "the specification is silent in regard to its substrate or its biological function" (the Advisory Action at page 5). The Advisory Action goes on to cite articles by Walker *et al.* (Cellular and Molecular Life Sciences 58:596-624, 2001) and Caughey (Am. R. Respir. Crit. Care. Med. 150:5138-5142, 1994) to show that proteases have different substrates and different specific biological roles, and concludes that therefore "further research" (the Advisory Action at page 5) would be required in order for the skilled artisan to use the presently claimed sequence. However, this argument is thwarted by the fact that expression profiling (as well as the utilities discussed above) does not require a knowledge of the function of the particular nucleic acid on the chip - rather the gene chip indicates which DNA fragments are expressed at greater or lesser levels in two or more particular tissue types. Skilled artisans already have used and continue to use sequences such as Appellants in gene chip applications without further experimentation. Appellants respectfully point out that this is exactly how most gene chip applications are carried out. Furthermore, the fact that additional information concerning the presently claimed sequence might make it even more useful in certain gene chip embodiments does not mean that the use of Appellants' sequence to track gene expression on a gene chip is not specific (*Carl Zeiss Stiftung v. Renishaw PLC, supra*). Therefore, this argument also fails to support the alleged lack of utility of the presently claimed compositions.

Clearly, persons of skill in the art, as well as venture capitalists and investors, readily recognize the

utility, both scientific and commercial, of genomic data in general, and specifically human genomic data. Billions of dollars have been invested in the human genome project, resulting in useful genomic data (see, *e.g.*, Venter *et al.*, *supra*; **Exhibit B**). The results have been a stunning success as the utility of human genomic data has been widely recognized as a great gift to humanity (see, *e.g.*, Jasny and Kennedy, 2001, *Science* 291:1153; **Exhibit K**). Clearly, the usefulness of human genomic data, such as the presently claimed nucleic acid molecules, is substantial and credible (worthy of billions of dollars and the creation of numerous companies focused on such information) and well-established (the utility of human genomic information has been clearly understood for many years).

Importantly, it has been clearly established that a statement of utility in a specification must be accepted absent reasons why one skilled in the art would have reason to doubt the objective truth of such statement. *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA, 1974; “*Langer*”); *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA, 1971). As clearly set forth in *Langer*:

As a matter of Patent Office practice, a specification which contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented must be taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject matter unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope.

Langer at 297, emphasis in original. As set forth in the MPEP, “Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered ‘false’ by a person of ordinary skill in the art” (MPEP, Eighth Edition at 2100-40, emphasis added). Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101.

Furthermore, regarding the utility requirements under 35 U.S.C. § 101, the Federal Circuit has clearly stated “(t)he threshold of utility is not high: An invention is ‘useful’ under section 101 if it is capable of providing some identifiable benefit.” *Juicy Whip Inc. v. Orange Bang Inc.*, 185 F.3d 1364, 51 USPQ2d 1700 (Fed. Cir. 1999) (citing *Brenner v. Manson*, 383 U.S. 519, 534 (1966)). Additionally, the Federal Circuit has stated that “(t)o violate § 101 the claimed device must be totally incapable of achieving a useful result.” *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571, 24 USPQ2d 1401 (Fed. Cir. 1992), emphasis added. *Cross v. Iizuka* (753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985); “*Cross*”) states “any utility of the claimed compounds is sufficient to satisfy 35 U.S.C.

§ 101". *Cross* at 748, emphasis added. Indeed, the Federal Circuit recently emphatically confirmed that "anything under the sun that is made by man" is patentable (*State Street Bank & Trust Co. v. Signature Financial Group Inc.*, 149 F.3d 1368, 47 USPQ2d 1596, 1600 (Fed. Cir. 1998), citing the U.S. Supreme Court's decision in *Diamond vs. Chakrabarty*, 447 U.S. 303, 206 USPQ 193 (U.S., 1980)). Thus, based on the relevant case law, the present claims clearly meet the requirements of 35 U.S.C. § 101.

Finally, While Appellants are well aware of the new Utility Guidelines set forth by the USPTO, Appellants respectfully point out that the current rules and regulations regarding the examination of patent applications is and always has been the patent laws as set forth in 35 U.S.C. and the patent rules as set forth in 37 C.F.R., not the Manual of Patent Examination Procedure or particular guidelines for patent examination set forth by the USPTO. Furthermore, it is the job of the judiciary, not the USPTO, to interpret these laws and rules. Appellants are unaware of any significant recent changes in either 35 U.S.C. § 101, or in the interpretation of 35 U.S.C. § 101 by the Supreme Court or the Federal Circuit that is in keeping with the new Utility Guidelines set forth by the USPTO. This is underscored by numerous patents that have been issued over the years that claim nucleic acid fragments that do not comply with the new Utility Guidelines. As examples of such issued U.S. Patents, the Board is invited to review U.S. Patent Nos. 5,817,479 (**Exhibit L**), 5,654,173 (**Exhibit M**), and 5,552,281 (**Exhibit N**; each of which claims short polynucleotides), and recently issued U.S. Patent No. 6,340,583 (**Exhibit O**; which includes no working examples), none of which contain examples of the "real-world" utilities that the Examiner seems to be requiring. As issued U.S. Patents are presumed to meet all of the requirements for patentability, including 35 U.S.C. §§ 101 and 112, first paragraph (see Section VIII(B), below), Appellants submit that the present polynucleotides must also meet the requirements of 35 U.S.C. § 101. While Appellants understand that each application is examined on its own merits, Appellants are unaware of any changes to 35 U.S.C. § 101, or in the interpretation of 35 U.S.C. § 101 by the Supreme Court or the Federal Circuit, since the issuance of these patents that render the subject matter claimed in these patents, which is similar to the subject matter in question in the present application, as suddenly non-statutory or failing to meet the requirements of 35 U.S.C. § 101. Thus, holding Appellants to a different standard of utility would be arbitrary and capricious, and, like other clear violations of due process, cannot stand.

For each of the foregoing reasons, Appellants submit that the rejection of claims 1-8 under 35 U.S.C. § 101 must be overruled.

B. Are Claims 1-8 Unusable Due to a Lack of Patentable Utility?

The Final Action next rejects claims 1-8 under 35 U.S.C. § 112, first paragraph, since allegedly one skilled in the art would not know how to use the invention, as the invention allegedly is not supported by either a clear asserted utility or a well-established utility.

The arguments detailed above in Section VIII(A) concerning the utility of the presently claimed sequences are incorporated herein by reference. As the Federal Circuit and its predecessor have determined that the utility requirement of Section 101 and the how to use requirement of Section 112, first paragraph, have the same basis, specifically the disclosure of a credible utility (*In re Brana, supra*; *In re Jolles*, 628 F.2d 1322, 1326 n.11, 206 USPQ 885, 889 n.11 (CCPA 1980); *In re Fouché*, 439 F.2d 1237, 1243, 169 USPQ 429, 434 (CCPA 1971)), Appellants submit that as claims 1-8 have been shown to have “a specific, substantial, and credible utility”, as detailed in Section VIII(A) above, the present rejection of claims 1-8 under 35 U.S.C. § 112, first paragraph, cannot stand.

Appellants therefore submit that the rejection of claims 1-8 under 35 U.S.C. § 112, first paragraph, must be overruled.

C. Is Claim 2 Indefinite?

The Final Action next rejected claim 2 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the invention.

The Final Action rejects claim 2 as allegedly indefinite based on the term “sequence” in relation to nucleic acid hybridization, since “(h)ybridization occurs only between molecules” (the Final Action at page 6), and the term “the complement thereof”, since this term is allegedly indefinite “for reasons of record” (the Final Action at page 6). First, Appellants stress that “a claim need not ‘describe’ the invention, such description being the role of the disclosure”. *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986). Appellants respectfully point out that the skilled artisan would

clearly understand how the nucleotide sequence could hybridize, within the parameters set forth in claim 2, and would also understand that the skilled artisan would understand the term “the complement” (as clearly opposed to “a” complement) to refer to the complete complement of SEQ ID NO:1. The claims are therefore sufficiently definite when read in light of the specification, which reasonably apprises those skilled in the art both of the utilization and scope of the invention. *Shatterproof Glass Corp. v. Libbey Owens Ford Co.*, 225 USPQ 634, 641 (Fed. Cir. 1985); *Miles Laboratories, Inc. v. Shandon*, 997 F.2d 870, 875, 27 USPQ2d 1123, 1126 (Fed. Cir. 1993); *Union Pacific Resources Co. v. Chesapeake Energy Corp.*, 236 F.3d 684, 692, 57 USPQ2d 1293, 1297 (Fed. Cir. 2001); *North American Vaccine, Inc. v. American Cyanamid Co.*, F.3d 1571, 1579, 28 USPQ2d 1333, 1339 (Fed. Cir. 1993); *Hybritech, Inc. v. Monoclonal Antibodies*, 802 F.2d 1367, 1385, 231 USPQ 81, 94-95 (Fed. Cir. 1986).

More importantly, however, Appellants submit that the United States Patent and Trademark Office itself finds this exact language to meet the requirements of 35 U.S.C. § 112, second paragraph, as evidenced at least by issued U.S. Patent Nos. 6,531,309 (**Exhibit P**), 6,511,840 (**Exhibit Q**), 6,476,210 (**Exhibit R**), 6,465,632 (**Exhibit S**), 6,462,186 (**Exhibit T**), 6,448,388 (**Exhibit U**), 6,444,456 (**Exhibit V**), 6,444,153 (**Exhibit W**) and 6,403,784 (**Exhibit X**), each of which each of which is assigned to the same entity as the present application and contains the exact same language that the Examiner finds indefinite in the present case. As issued U.S. Patents are presumed to meet all of the requirements for patentability, including 35 U.S.C. § 112, second paragraph, Appellants submit that claim 2 must also meet the requirements of 35 U.S.C. § 112, second paragraph. Holding Appellants to a different standard of definiteness would be arbitrary and capricious, and, like other clear violations of due process, cannot stand.

For each of the foregoing reasons, Appellants submit that the rejection of claim 2 under 35 U.S.C. § 112, second paragraph, must be overruled.

D. Do Claims 1, 5 and 8 Lack Sufficient Written Description?

The Final Action next rejected claims 1, 5 and 8 under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed,

had possession of the claimed invention.

The Examiner seems to be requiring that the function of each of the members of the genus be known in order to satisfy the written description requirement. However, the Examiner's stated position completely misreads the written description requirement. The repeated citations of Broun *et al.* (*supra*), Van de Loo *et al.* (*supra*), Seffernick *et al.* (*supra*), and Witkowski *et al.* (*supra*), each of which were dealt with in Section VIII(A), above, for the proposition that changes in protein sequence can lead to changes in protein function, are **completely irrelevant** to the present question of compliance with 35 U.S.C. § 112, first paragraph. As set forth in the response to the Second Action and the response to the Final Action, the relevant section of the written description guidelines is herein reproduced with numbers corresponding to the ways in which the written description requirement can be satisfied: (1) by actual reduction to practice, (2) reduction to drawings, or (3) by disclosure of relevant, identifying characteristics, i.e., (4) structure or other physical and/or chemical properties, (5) by functional characteristics coupled with a known or disclosed correlation between function and structure, or (6) by a combination of such identifying characteristics. Thus, the written description requirements can be satisfied by (1), (2), or (3), and part (3) can be satisfied by (4), (5), or (6). Appellants submit that claim 1 provides "structure or other physical or chemical properties", specifically, the **nucleotide sequence itself**. There is **no requirement** within section (4) for functional characteristics, this being included in sections (5) and (6) **only**. Thus, since claims 1, 5 and 8 satisfy section (3) by satisfying section (4), claims 1, 5 and 8 **must** meet the written description requirement.

35 U.S.C. § 112, first paragraph, requires that the specification contain a written description of the invention. The Federal Circuit in *Vas-Cath Inc. v. Mahurkar* (19 USPQ2d 1111 (Fed. Cir. 1991); "*Vas-Cath*") held that an "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*." *Vas-Cath*, at 1117, emphasis in original. However, it is important to note that the above finding uses the terms reasonable clarity to those skilled in the art. Further, the Federal Circuit in *In re Gosteli* (10 USPQ2d 1614 (Fed. Cir. 1989); "*Gosteli*") held:

Although [the applicant] does not have to describe exactly the subject matter claimed, . . . the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.

Gosteli at 1618, emphasis added. Additionally, *Utter v. Hiraga* (6 USPQ2d 1709 (Fed. Cir. 1988); “*Utter*”), held “(a) specification may, within the meaning of 35 U.S.C. § 112 ¶1, contain a written description of a broadly claimed invention without describing all species that claim encompasses” (*Utter*, at 1714). Therefore, all Appellants must do to comply with 35 U.S.C. § 112, first paragraph, is to convey the invention with reasonable clarity to the skilled artisan.

Further, the Federal Circuit has held that an adequate description of a chemical genus “requires a precise definition, such as by structure, formula, chemical name or physical properties” sufficient to distinguish the genus from other materials. *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993; “*Fiers*”). *Fiers* goes on to hold that the “application satisfies the written description requirement since it sets forth the . . . nucleotide sequence” (*Fiers* at 1607). In other words, provision of a structure and formula - the nucleotide sequence - renders the application in compliance with 35 U.S.C. § 112, first paragraph.

More recently, the standard for complying with the written description requirement in claims involving chemical materials has been explicitly set forth by the Federal Circuit:

In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus. *Regents of Univ. of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

Thus, a claim describing a genus of nucleic acids by structure, formula, chemical name or physical properties sufficient to allow one of ordinary skill in the art to distinguish the genus from other materials meets the written description requirement of 35 U.S.C. § 112, first paragraph. As further elaborated by the Federal Circuit in *Regents of Univ. of California v. Eli Lilly and Co.*:

In claims to genetic material ... a generic statement such as ‘vertebrate insulin cDNA’ or ‘mammalian insulin cDNA’, without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function. It does not specifically define any of the genes that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art cannot, as one can do with a fully described genus, visualize or recognize the identity of members of the genus. (Emphasis added)

Thus, as opposed to the situation set forth in *Regents of Univ. of California v. Eli Lilly and Co.* and *Fiers*, the nucleic acid sequences of the present invention are not distinguished on the basis of function, or a method of isolation, but in fact are distinguished by structural features - a chemical formula, *i.e.*, the *sequence itself*.

Using the nucleic acid sequences of the present invention (as set forth in the Sequence Listing), the skilled artisan would readily be able to distinguish the claimed nucleic acids from other materials on the basis of the specific structural description provided. Polynucleotides comprising at least 24 contiguous bases from SEQ ID NO:1 are within the genus of the instant claims, while those that lack this structural feature lie outside the genus. Importantly, the Final Action admitted that claims 1, 5 and 8 do in fact include a distinguishing feature, specifically, that the nucleic acid molecule must include “at least 24 consecutive nucleotides of the polynucleotide of SEQ ID NO:1” (the Final Action at page 7). Additionally, the Advisory Action the Examiner agrees “that the claimed genus of polynucleotides is defined in structural terms” (the Advisory Action at page 7). Appellants respectfully point out that this is all that is required of claims 1, 5 and 8 to meet the written description requirement of 35 U.S.C. § 112, first paragraph.

For each of the foregoing reasons, Appellants submit that the rejection of claims 1, 5 and 8 under 35 U.S.C. § 112, first paragraph, must be overruled.

E. Are Claims 1, 5 and 8 Enabled?

The Final Action next rejected claims 1, 5 and 8 under 35 U.S.C. § 112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention.

The Final Action stated that “this enablement rejection was applied due to the lack of information as to how one of skill in the art can reasonably make and use the polynucleotides, as encompassed by the claims” (the Final Action at page 9; emphasis in original). The Final Action once again contends that the specification provides insufficient guidance regarding the biological function or activity of certain of the claimed compositions. As set forth in Section VIII(D), above, the repeated citations of Broun *et al.* (*supra*) and Van de Loo *et al.* (*supra*), Seffernick *et al.* (*supra*), and Witkowski *et al.* (*supra*), for the

proposition that changes in protein sequence can lead to changes in protein function, are once again **completely irrelevant** to the present question of compliance with 35 U.S.C. § 112, first paragraph. Importantly, such an enablement standard conflicts with established patent law.

Appellants point out that significant commercial exploitation of nucleic acid sequences requires no more information than the nucleic acid sequence itself. Applications ranging from gene expression analysis or profiling (utilizing, for example, arrays of short, overlapping or non-overlapping, oligonucleotides and DNA chips, as described in Section VIII(A), above) to chromosomal mapping (utilizing, for example, short oligonucleotide probes or full length DNA sequences, as described in Section VIII(A), above) are practiced utilizing nucleic acid sequences and techniques that are well-known to those of skill in the art. The widespread commercial exploitation of nucleic acid sequence information points to the level of skill in the art, and the enablement provided by disclosures such as the present specification, which include specific nucleic acid sequences and guidance regarding the various uses of such sequences. Thus, the skilled artisan can clearly make and use the claimed polynucleotides, which is **all that is required** to meet the enablement requirement under 35 U.S.C. § 112, first paragraph.

The Examiner states that the present invention could not be practiced without “undue experimentation” (the Final Action bridging pages 10 and 11). However, it is important to remember that in assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is “undue”, not “experimentation”. *In re Angstadt and Griffin, supra*. In *In re Wands (supra; “Wands”)*, the P.T.O. took the position that the applicant failed to demonstrate that the disclosed biological processes of immunization and antibody selection could reproducibly result in a useful biological product (antibodies from hybridomas) within the scope of the claims. In its decision overturning the P.T.O.’s rejection, the Federal Circuit found that Wands’ demonstration of success in four out of nine cell lines screened was sufficient to support a conclusion of enablement. The court emphasized that the need for some experimentation requiring, *e.g.*, production of the biological material followed by routine screening, was not a basis for a finding of non-enablement, stating:

Disclosure in application for the immunoassay method patent does not fail to meet enablement requirement of 35 USC 112 by requiring ‘undue experimentation,’ even though production of monoclonal antibodies necessary to practice invention first requires

production and screening of numerous antibody producing cells or 'hybridomas,' since practitioners of art are prepared to screen negative hybridomas in order to find those that produce desired antibodies, since in monoclonal antibody art one 'experiment' is not simply screening of one hybridoma but rather is entire attempt to make desired antibody, and since record indicates that amount of effort needed to obtain desired antibodies is not excessive, in view of Applicants' success in each attempt to produce antibody that satisfied all claim limitations.

Wands at 1400. Thus, the need for some experimentation does not render the claimed invention unpatentable under 35 U.S.C. § 112, first paragraph. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Angstadt and Griffin, supra; Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd., supra.*

The Final Action questioned the teaching and guidance in the specification for certain aspects of the present invention. However, as discussed above, this requirement is completely misplaced. There is sufficient knowledge and technical skill in the art for a skilled artisan to be able to make and use the claimed DNA species in a number of different aspects of the invention entirely without further details in a patent specification. For example, it is not unreasonable to expect a Ph.D. level molecular biologist to be able to use the disclosed sequence to design oligonucleotide probes and primers and use them in, for example, PCR based screening and detection methods to obtain the described sequences and/or determine tissue expression patterns. Nevertheless, the present specification provides highly detailed descriptions of techniques that can be used to accomplish many different aspects of the claimed invention, including recombinant expression, site-specific mutagenesis, *in situ* hybridization, and large scale nucleic acid screening techniques, and properly incorporates by reference a montage of standard texts into the specification, such as Sambrook *et al.* (*Molecular Cloning, A Laboratory Manual*) and Ausubel *et al.* (*Current Protocols in Molecular Biology*) to provide even further guidance to the skilled artisan. Incorporation of material into the specification by reference is proper. *Ex parte Schwarze*, 151 USPQ 426 (PTO Bd. App. 1966). The § 112, first paragraph rejection is thus *prima facie* improper:

As a matter of patent office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first

paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Marzocchi, supra, emphasis as in original. In any event, an alleged lack of express teaching is insufficient to support a first paragraph rejection where one of skill in the art would know how to perform techniques required to perform at least one aspect of the invention. As a matter of law, it is well settled that a patent need not disclose what is well known in the art. *In re Wands, supra*. In fact, it is preferable that what is well known in the art be omitted from the disclosure. *Hybritech, Inc. v. Monoclonal Antibodies, Inc., supra*. As standard molecular biological techniques are routine in the art, such protocols do not need to be described in detail in the specification.

As discussed *In re Brana (supra, "Brana")*, the Federal Circuit admonished the P.T.O. for confusing "the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption". *Brana* at 1442. Furthermore, a specification "need describe the invention only in such detail as to enable a person skilled in the most relevant art to make and use it." *In re Naquin*, 158 USPQ 317, 319 (CCPA 1968); emphasis added. The present claims are thus enabled as they are supported by a specification that provides sufficient description to enable the skilled person to make and use the invention as claimed. Appellants stress that enablement must be analyzed, not in a vacuum, but "as it would be interpreted by one possessing the ordinary level of skill in the pertinent art." *In re Moore*, 169 USPQ 236, 238 (CCPA 1971).

It has long been established that claims are enabled by defining any practical use. *In re Nelson*, 126 USPQ 242 (CCPA 1960); *Cross v. Iizuka, supra*. "The enablement requirement is met if the description enables any mode of making and using the invention." *Johns Hopkins Univ. v. CellPro, Inc.*, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998), citing *Engel Indus., Inc. v. Lockformer Co.*, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991). As described in detail above, the specification details numerous applications in which claimed nucleotide sequences can be used, for example, to track gene expression using gene chips. Further, since public domain nucleotide sequences that have not been associated with any particular biological function, let alone validated as coding sequences, are used every day in gene chip applications, it defies logic that undue experimentation would be required to use the presently described nucleotide sequences, which have been biologically validated as coding sequences, in the very same gene chip

applications.

Appellants therefore submit that the rejection of claims 1, 5 and 8 under 35 U.S.C. § 112, first paragraph, must be overruled.

IX. APPENDIX

The claims involved in this appeal are as follows:

1. (Amended) An isolated nucleic acid molecule comprising at least 24 contiguous bases of nucleotide sequence from SEQ ID NO:1.
2. (Amended) An isolated nucleic acid molecule comprising a nucleotide sequence that:
 - (a) encodes the amino acid sequence shown in SEQ ID NO:2; and
 - (b) hybridizes under highly stringent conditions to the nucleotide sequence of SEQ ID NO:1 or the complement thereof.
3. (Amended) An isolated nucleic acid molecule according to Claim 1 wherein said nucleotide sequence is a cDNA sequence.
4. An isolated nucleic acid molecule according to Claim 3 encoding the amino acid sequence described in SEQ ID NO:2.
5. A recombinant expression vector comprising the isolated nucleic acid molecule of claim 1.
6. The recombinant expression vector of claim 5, wherein said isolated nucleic acid molecule encodes the amino acid sequence of SEQ ID NO:2.
7. The recombinant expression vector of claim 6, wherein said isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:1.
8. A host cell comprising the recombinant expression vector of claim 5.

X. CONCLUSION

Appellants respectfully submit that, in light of the foregoing arguments, the Final Action's conclusion that claims 1-8 lack a patentable utility and are unusable by the skilled artisan due to a lack of patentable utility, that claim 2 is indefinite, and that claims 1, 5 and 8 lack sufficient written description and are not enabled, are unwarranted. It is therefore requested that the Board overturn the Final Action's rejections.

Respectfully submitted,

July 3, 2003

Date

David W. Hibler

David W. Hibler
Agent For Appellants

Reg. No. 41,071

LEXICON GENETICS INCORPORATED
8800 Technology Forest Place
The Woodlands, TX 77381
(281) 863-3399



24231

PATENT TRADEMARK OFFICE

TABLE OF AUTHORITIES

CASES

<i>Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.</i> , 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)	9, 24
<i>Brooktree Corp. v. Advanced Micro Devices, Inc.</i> , 977 F.2d 1555, 1571, 24 USPQ2d 1401 (Fed. Cir. 1992)	16
<i>Carl Zeiss Stiftung v. Renishaw PLC</i> , 20 USPQ2d 1101 (Fed. Cir. 1991) (citing <i>Envirotech Corp. v. Al George, Inc.</i> , 221 USPQ 473, 480 (Fed. Cir. 1984))	6, 7, 11, 15
<i>Cross v. Iizuka</i> , 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985)	16, 17, 25
<i>Diamond vs. Chakrabarty</i> , 447 U.S. 303, 206 USPQ 193 (U.S., 1980)	17
<i>Ex parte Schwarze</i> , 151 USPQ 426 (PTO Bd. App. 1966)	24
<i>Fiers v. Revel</i> , 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993)	21, 22
<i>Hoffman v. Klaus</i> , 9 USPQ2d 1657 (Bd. Pat. App. & Inter. 1988)	9
<i>Hybritech, Inc. v. Monoclonal Antibodies</i> , 802 F.2d 1367, 1385, 231 USPQ 81, 94-95 (Fed. Cir. 1986)	19, 25
<i>In re Angstadt and Griffin</i> , 537 F.2d 498, 190 USPQ 214 (CCPA 1976)	8, 9, 23, 24

<i>In re Brana</i> , 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995)	7, 8, 18, 25
<i>In re Fouche</i> , 439 F.2d 1237, 1243, 169 USPQ 429, 434 (CCPA 1971)	18
<i>In re Gosteli</i> , 872 F.2d 1008, 10 USPQ2d 1614 (Fed. Cir. 1989)	20, 21
<i>In re Gottlieb</i> , 328 F.2d 1016, 140 USPQ 665 (CCPA 1964)	9
<i>In re Jolles</i> , 628 F.2d 1322, 1326 n.11, 206 USPQ 885, 889 n.11 (CCPA 1980)	18
<i>In re Langer</i> , 503 F.2d 1380, 183 USPQ 288 (CCPA 1974)	16
<i>In re Malachowski</i> , 530 F.2d 1402, 189 USPQ 432 (CCPA 1976)	9
<i>In re Marzocchi</i> , 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA, 1971)	16, 24, 25
<i>In re Moore</i> , 439 F.2d 1232, 169 USPQ 236 (CCPA 1971)	25
<i>In re Naquin</i> , 158 USPQ 317, 319 (CCPA 1968)	25
<i>In re Nelson</i> , 280 F.2d 172, 126 USPQ 242 (CCPA 1960)	25
<i>In re Wands</i> , 858 F.2d 731, 8 USPQ 2d 1400 (Fed. Cir. 1988)	6, 9, 23-25
<i>Johns Hopkins Univ. v. CellPro, Inc.</i> , 152 F.3d 1342, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) (citing <i>Engel Indus., Inc. v. Lockformer Co.</i> , 946 F.2d 1528, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991))	25

<i>Juicy Whip Inc. v. Orange Bang Inc.</i> , 185 F.3d 1364, 51 USPQ2d 1700 (Fed. Cir. 1999) (citing <i>Brenner v. Manson</i> , 383 U.S. 519, 534 (1966))	16
<i>Miles Laboratories, Inc. v. Shandon</i> , 997 F.2d 870, 27 USPQ2d 1123 (Fed. Cir. 1993)	19
<i>North American Vaccine, Inc. v. American Cyanamid Co.</i> , F.3d 1571, 1579, 28 USPQ2d 1333, 1339 (Fed. Cir. 1993)	19
<i>Orthokinetics, Inc. v. Safety Travel Chairs, Inc.</i> , 1 USPQ2d 1081, 1088 (Fed. Cir. 1986)	18
<i>Raytheon Co. v. Roper Corp.</i> , 724 F.2d 951, 220 USPQ 592 (Fed. Cir. 1983)	9
<i>Regents of Univ. of California v. Eli Lilly and Co.</i> , 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997)	21, 22
<i>Shatterproof Glass Corp. v. Libbey Owens Ford Co.</i> , 758 F.2d 613, 225 USPQ 634 (Fed. Cir. 1985)	19
<i>State Street Bank & Trust Co. v. Signature Financial Group Inc.</i> , 149 F.3d 1368, 47 USPQ2d 1596, 1600 (Fed. Cir. 1998)	17
<i>Union Pacific Resources Co. v. Chesapeake Energy Corp.</i> , 236 F.3d 684, 692, 57 USPQ2d 1293, 1297 (Fed. Cir. 2001)	19
<i>Utter v. Hiraga</i> , 845 F.2d 993, 6 USPQ2d 1709 (Fed. Cir. 1988)	21
<i>Vas-Cath Inc. v. Mahurkar</i> , 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991)	20

STATUTES

35 U.S.C. § 101 2-4, 7-10, 14-18

35 U.S.C. § 112 2-4, 8, 17-26

A

This Page Blank (uspto)

Query= SEQ ID NO:1
(1041 letters)

Sequences producing significant alignments:

	Score (bits)	E Value
--	-----------------	------------

AC104819.4.1.129203	766	0.0
---------------------	-----	-----

>AC104819.4.1.129203
Length = 129203

Score = 766 bits (386), Expect = 0.0
Identities = 390/392 (99%)
Strand = Plus / Plus

Query: 650	agggtgattctggagggcctctgtcgtgtcacattgatgggtgatggatccagacaggag	709
Sbjct: 46368	agggtgattctggagggcctctgtcgtgtcacattgatgggtgatggatccagacaggag	46427

Query: 710	tagtaagctggggattagaatgtggtaaattctcttcctggagctacaccaatgtaatct	769
Sbjct: 46428	tagtaagctggggattagaatgtggtaaattctcttcctggagctacaccaatgtaatct	46487

Query: 770	actacaaaaatggattaatgccactatttcaagagccaacaatctagacttctctgact	829
Sbjct: 46488	actacaaaaatggattaatgccactatttcaagagccaacaatctagacttctctgact	46547

Query: 830	tcttggtccctattgtcctactctctctggctctcctgygtccctcctgtgcctttggac	889
Sbjct: 46548	tcttggtccctattgtcctactctctctggctctcctgcgtccctcctgtgcctttggac	46607

Query: 890	ctaacactatacacagagtaggcactgtagctgaagctggttgcttgcatcacagggctggg	949
Sbjct: 46608	ctaacactatacacagagtaggcactgtagctgaagctggttgcttgcatcacagggctggg	46667

Query: 950	aagagaatgcatggagatttagtcccaggggcagagaactcacaggagagccactgctaa	1009
Sbjct: 46668	aagagaatgcatggagatttagtcccaggggcagataactcacaggagagccactgctaa	46727

Query: 1010	ccctgggtgactttattttacaatttgaaatga	1041
Sbjct: 46728	ccctgggtgactttattttacaatttgaaatga	46759

Score = 524 bits (264), Expect = e-146
Identities = 265/266 (99%)
Strand = Plus / Plus

Query: 216 gacctggactactttttcatatactgtgtggctaggatcgattacagtaggtgactcaag 275
|||||
Sbjct: 37400 gacctggactactttttcatatactgtgtggctaggatcgattacagtaggtgactcaag 37459

Query: 276 gaaacgtgtgaagtactacgtgtccaaaatcgatccatcccaagtaggaagataacaac 335
|||||
Sbjct: 37460 gaaacgtgtgaagtactacgtgtccaaaatcgatccatcccaagtaggaagataacaac 37519

Query: 336 ggcagacrtcgcttgttgaaactgtcctctcaagtcaccttcacttctgccatcctgcc 395
|||||
Sbjct: 37520 ggcagacgtcgcttgttgaaactgtcctctcaagtcaccttcacttctgccatcctgcc 37579

Query: 396 tatttgcttgcccagtggtcacaaagcagttggcaattccacccttttgttgggtgaccgg 455
|||||
Sbjct: 37580 tatttgcttgcccagtggtcacaaagcagttggcaattccacccttttgttgggtgaccgg 37639

Query: 456 atggggaaaagttaaggaaagttag 481
|||||
Sbjct: 37640 atggggaaaagttaaggaaagttag 37665

Score = 345 bits (174), Expect = 3e-92
Identities = 174/174 (100%)
Strand = Plus / Plus

Query: 479 cagatagagattaccattctgcccttcaggaagcagaagtacccattattgaccgccagg 538
|||||
Sbjct: 38366 cagatagagattaccattctgcccttcaggaagcagaagtacccattattgaccgccagg 38425

Query: 539 cttgtgaacagctctacaatcccatcggtatcttcttgccagcactggagccagtcacatca 598
|||||
Sbjct: 38426 cttgtgaacagctctacaatcccatcggtatcttcttgccagcactggagccagtcacatca 38485

Query: 599 aggaagacaagatttgtgctggtgatactcaaaacatgaaggatagttgcaagg 652
|||||
Sbjct: 38486 aggaagacaagatttgtgctggtgatactcaaaacatgaaggatagttgcaagg 38539

Score = 322 bits (162), Expect = 4e-85
Identities = 169/171 (98%), Gaps = 1/171 (0%)
Strand = Plus / Plus

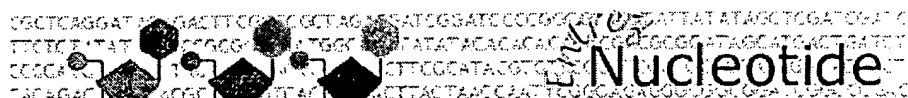
Query: 47 tctc-agtgtgtgggcaacctgtatactccagccgcgttgtaggtggccaggatgctgct 105
|||||
Sbjct: 35041 tctctagtgtgtgggcaacctgtatactccagccgcgttgtaggtggccaggatgctgct 35100

Query: 106 gcagggcgctggccttggcaggtcagcctacactttgaccacaactttatctatggaggt 165
|||||
Sbjct: 35101 gcagggcgctggccttggcaggtcagcctacactttgaccacaactttatctgtggaggt 35160

Query: 166 tccctcgtcagtgagaggttgatactgacagcagcacactgcatacaaccg 216
|||||
Sbjct: 35161 tccctcgtcagtgagaggttgatactgacagcagcacactgcatacaaccg 35211

Score = 103 bits (52), Expect = 2e-19
Identities = 52/52 (100%)
Strand = Plus / Plus

Query: 1 atgggccctgctggctgtgccttcacgctgctccttctgctggggatctcag 52
|||||
Sbjct: 32425 atgggccctgctggctgtgccttcacgctgctccttctgctggggatctcag 32476



PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

OMIM

Boo

Search

for

Go

Clear

Limits

Preview/Index

History

Clipboard

Details

Display

default

Show:

20

Send to

File

Get Subsequence

☐ 1: AC104819. Homo sapiens BAC ...[gi:20177800]

Links

LOCUS AC104819 129203 bp DNA linear PRI 29-MAY-2002
 DEFINITION Homo sapiens BAC clone RP11-731D1 from 4, complete sequence.
 ACCESSION AC104819
 VERSION AC104819.4 GI:20177800
 KEYWORDS HTG.
 SOURCE Homo sapiens (human)
 ORGANISM Homo sapiens
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 129203)
 AUTHORS Sulston, J.E. and Waterston, R.
 TITLE Toward a complete human genome sequence
 JOURNAL Genome Res. 8 (11), 1097-1108 (1998)
 MEDLINE 99063792
 PUBMED 9847074

REFERENCE 2 (bases 1 to 129203)
 AUTHORS Pearman, C., Abbott, A. and Spalding, L.
 TITLE The sequence of Homo sapiens BAC clone RP11-731D1
 JOURNAL Unpublished (2001)

REFERENCE 3 (bases 1 to 129203)
 AUTHORS Waterston, R.H.
 TITLE Direct Submission
 JOURNAL Submitted (21-DEC-2001) Genome Sequencing Center, Washington
 University School of Medicine, 4444 Forest Park Parkway, St. Louis,
 MO 63108, USA

REFERENCE 4 (bases 1 to 129203)
 AUTHORS Waterston, R.H.
 TITLE Direct Submission
 JOURNAL Submitted (20-MAR-2002) Genome Sequencing Center, Washington
 University School of Medicine, 4444 Forest Park Parkway, St. Louis,
 MO 63108, USA

REFERENCE 5 (bases 1 to 129203)
 AUTHORS Waterston, R.H.
 TITLE Direct Submission
 JOURNAL Submitted (18-APR-2002) Genome Sequencing Center, Washington
 University School of Medicine, 4444 Forest Park Parkway, St. Louis,
 MO 63108, USA

REFERENCE 6 (bases 1 to 129203)
 AUTHORS Waterston, R.
 TITLE Direct Submission
 JOURNAL Submitted (29-MAY-2002) Department of Genetics, Washington
 University, 4444 Forest Park Avenue, St. Louis, Missouri 63108, USA

COMMENT On Apr 18, 2002 this sequence version replaced gi:19551227.
 ----- Genome Center
 Center: Washington University Genome Sequencing Center
 Center code: WUGSC
 Web site: <http://genome.wustl.edu/gsc>

B

This Page Blank (uspto)

The Sequence of the Human Genome

J. Craig Venter,^{1*} Mark D. Adams,¹ Eugene W. Myers,¹ Peter W. Li,¹ Richard J. Mural,¹
 Granger G. Sutton,¹ Hamilton O. Smith,¹ Mark Yandell,¹ Cheryl A. Evans,¹ Robert A. Holt,¹
 Jeannine D. Gocayne,¹ Peter Amanatides,¹ Richard M. Ballew,¹ Daniel H. Huson,¹
 Jennifer Russo Wortman,¹ Qing Zhang,¹ Chinnappa D. Kodira,¹ Xiangqun H. Zheng,¹ Lin Chen,¹
 Marian Skupski,¹ Gangadharan Subramanian,¹ Paul D. Thomas,¹ Jinghui Zhang,¹
 George L. Gabor Miklos,² Catherine Nelson,³ Samuel Broder,¹ Andrew G. Clark,⁴ Joe Nadeau,⁵
 Victor A. McKusick,⁶ Norton Zinder,⁷ Arnold J. Levine,⁷ Richard J. Roberts,⁸ Mel Simon,⁹
 Carolyn Slayman,¹⁰ Michael Hunkapiller,¹¹ Randall Bolanos,¹ Arthur Delcher,¹ Ian Dew,¹ Daniel Fasulo,¹
 Michael Flanigan,¹ Liliana Florea,¹ Aaron Halpern,¹ Sridhar Hannenhalli,¹ Saul Kravitz,¹ Samuel Levy,¹
 Clark Mobarry,¹ Knut Reinert,¹ Karin Remington,¹ Jane Abu-Threideh,¹ Ellen Beasley,¹ Kendra Biddick,¹
 Vivien Bonazzi,¹ Rhonda Brandon,¹ Michele Cargill,¹ Ishwar Chandramouliswaran,¹ Rosane Charlab,¹
 Kabir Chaturvedi,¹ Zuoming Deng,¹ Valentina Di Francesco,¹ Patrick Dunn,¹ Karen Eilbeck,¹
 Carlos Evangelista,¹ Andrei E. Gabrielian,¹ Weiniu Gan,¹ Wangmao Ge,¹ Fangcheng Gong,¹ Zhiping Gu,¹
 Ping Guan,¹ Thomas J. Heiman,¹ Maureen E. Higgins,¹ Rui-Ru Ji,¹ Zhaoxi Ke,¹ Karen A. Ketchum,¹
 Zhongwu Lai,¹ Yiding Lei,¹ Zhenya Li,¹ Jiayin Li,¹ Yong Liang,¹ Xiaoying Lin,¹ Fu Lu,¹
 Gennady V. Merkulov,¹ Natalia Milshina,¹ Helen M. Moore,¹ Ashwinikumar K Naik,¹
 Vaibhav A. Narayan,¹ Beena Neelam,¹ Deborah Nusskern,¹ Douglas B. Rusch,¹ Steven Salzberg,¹²
 Wei Shao,¹ Bixiong Shue,¹ Jingtao Sun,¹ Zhen Yuan Wang,¹ Aihui Wang,¹ Xin Wang,¹ Jian Wang,¹
 Ming-Hui Wei,¹ Ron Wides,¹³ Chunlin Xiao,¹ Chunhua Yan,¹ Alison Yao,¹ Jane Ye,¹ Ming Zhan,¹
 Weiqing Zhang,¹ Hongyu Zhang,¹ Qi Zhao,¹ Liansheng Zheng,¹ Fei Zhong,¹ Wenyan Zhong,¹
 Shiaoping C. Zhu,¹ Shaying Zhao,¹² Dennis Gilbert,¹ Suzanna Baumhueter,¹ Gene Spier,¹
 Christine Carter,¹ Anibal Cravchik,¹ Trevor Woodage,¹ Feroze Ali,¹ Huijin An,¹ Aderonke Awe,¹
 Danita Baldwin,¹ Holly Baden,¹ Mary Barnstead,¹ Ian Barrow,¹ Karen Beeson,¹ Dana Busam,¹
 Amy Carver,¹ Angela Center,¹ Ming Lai Cheng,¹ Liz Curry,¹ Steve Danaher,¹ Lionel Davenport,¹
 Raymond Desilets,¹ Susanne Dietz,¹ Kristina Dodson,¹ Lisa Doup,¹ Steven Ferriera,¹ Neha Garg,¹
 Andres Gluecksmann,¹ Brit Hart,¹ Jason Haynes,¹ Charles Haynes,¹ Cheryl Heiner,¹ Suzanne Hladun,¹
 Damon Hostin,¹ Jarrett Houck,¹ Timothy Howland,¹ Chinyere Ibegwam,¹ Jeffery Johnson,¹
 Francis Kalush,¹ Lesley Kline,¹ Shashi Koduru,¹ Amy Love,¹ Felecia Mann,¹ David May,¹
 Steven McCawley,¹ Tina McIntosh,¹ Ivy McMullen,¹ Mee Moy,¹ Linda Moy,¹ Brian Murphy,¹
 Keith Nelson,¹ Cynthia Pfannkoch,¹ Eric Pratts,¹ Vinita Puri,¹ Hina Qureshi,¹ Matthew Reardon,¹
 Robert Rodriguez,¹ Yu-Hui Rogers,¹ Deanna Romblad,¹ Bob Ruhfel,¹ Richard Scott,¹ Cynthia Sitter,¹
 Michelle Smallwood,¹ Erin Stewart,¹ Renee Strong,¹ Ellen Suh,¹ Reginald Thomas,¹ Ni Ni Tint,¹
 Sukyee Tse,¹ Claire Vech,¹ Gary Wang,¹ Jeremy Wetter,¹ Sherita Williams,¹ Monica Williams,¹
 Sandra Windsor,¹ Emily Winn-Deen,¹ Keriellen Wolfe,¹ Jayshree Zaveri,¹ Karena Zaveri,¹
 Josep F. Abril,¹⁴ Roderic Guigó,¹⁴ Michael J. Campbell,¹ Kimmen V. Sjolander,¹ Brian Karlak,¹
 Anish Kejariwal,¹ Huaiyu Mi,¹ Betty Lazareva,¹ Thomas Hatton,¹ Apurva Narechania,¹ Karen Diemer,¹
 Anushya Muruganujan,¹ Nan Guo,¹ Shinji Sato,¹ Vineet Bafna,¹ Sorin Istrail,¹ Ross Lippert,¹
 Russell Schwartz,¹ Brian Walenz,¹ Shibu Yooseph,¹ David Allen,¹ Anand Basu,¹ James Baxendale,¹
 Louis Blick,¹ Marcelo Caminha,¹ John Carnes-Stine,¹ Parris Caulk,¹ Yen-Hui Chiang,¹ My Coyne,¹
 Carl Dahlke,¹ Anne Deslattes Mays,¹ Maria Dombroski,¹ Michael Donnelly,¹ Dale Ely,¹ Shiva Esparham,¹
 Carl Fosler,¹ Harold Gire,¹ Stephen Glanowski,¹ Kenneth Glasser,¹ Anna Glodek,¹ Mark Gorokhov,¹
 Ken Graham,¹ Barry Gropman,¹ Michael Harris,¹ Jeremy Heil,¹ Scott Henderson,¹ Jeffrey Hoover,¹
 Donald Jennings,¹ Catherine Jordan,¹ James Jordan,¹ John Kasha,¹ Leonid Kagan,¹ Cheryl Kraft,¹
 Alexander Levitsky,¹ Mark Lewis,¹ Xiangjun Liu,¹ John Lopez,¹ Daniel Ma,¹ William Majoros,¹
 Joe McDaniel,¹ Sean Murphy,¹ Matthew Newman,¹ Trung Nguyen,¹ Ngoc Nguyen,¹ Marc Nodell,¹
 Sue Pan,¹ Jim Peck,¹ Marshall Peterson,¹ William Rowe,¹ Robert Sanders,¹ John Scott,¹
 Michael Simpson,¹ Thomas Smith,¹ Arlan Sprague,¹ Timothy Stockwell,¹ Russell Turner,¹ Eli Venter,¹
 Mei Wang,¹ Meiyuan Wen,¹ David Wu,¹ Mitchell Wu,¹ Ashley Xia,¹ Ali Zandieh,¹ Xiaohong Zhu¹

Exhibit B
 LEX-0176-USA
 nucleotides Encoding the Same

Deco
 hum:
 for tl

—
 Cele
 2085
 Beach
 Geno
 9472
 versit
 USA.
 Univ
 Aven
 Univ
 tal, 6
 MD:
 York
 Engl
 USA.
 of T
 dens
 Med
 Hav
 850
 12T
 Cen
 Life
 Isra
 stitu
 Pon
 *Tc
 mai

A 2.91-billion base pair (bp) consensus sequence of the euchromatic portion of the human genome was generated by the whole-genome shotgun sequencing method. The 14.8-billion bp DNA sequence was generated over 9 months from 27,271,853 high-quality sequence reads (5.11-fold coverage of the genome) from both ends of plasmid clones made from the DNA of five individuals. Two assembly strategies—a whole-genome assembly and a regional chromosome assembly—were used, each combining sequence data from Celera and the publicly funded genome effort. The public data were shredded into 550-bp segments to create a 2.9-fold coverage of those genome regions that had been sequenced, without including biases inherent in the cloning and assembly procedure used by the publicly funded group. This brought the effective coverage in the assemblies to eightfold, reducing the number and size of gaps in the final assembly over what would be obtained with 5.11-fold coverage. The two assembly strategies yielded very similar results that largely agree with independent mapping data. The assemblies effectively cover the euchromatic regions of the human chromosomes. More than 90% of the genome is in scaffold assemblies of 100,000 bp or more, and 25% of the genome is in scaffolds of 10 million bp or larger. Analysis of the genome sequence revealed 26,588 protein-encoding transcripts for which there was strong corroborating evidence and an additional ~12,000 computationally derived genes with mouse matches or other weak supporting evidence. Although gene-dense clusters are obvious, almost half the genes are dispersed in low G+C sequence separated by large tracts of apparently noncoding sequence. Only 1.1% of the genome is spanned by exons, whereas 24% is in introns, with 75% of the genome being intergenic DNA. Duplications of segmental blocks, ranging in size up to chromosomal lengths, are abundant throughout the genome and reveal a complex evolutionary history. Comparative genomic analysis indicates vertebrate expansions of genes associated with neuronal function, with tissue-specific developmental regulation, and with the hemostasis and immune systems. DNA sequence comparisons between the consensus sequence and publicly funded genome data provided locations of 2.1 million single-nucleotide polymorphisms (SNPs). A random pair of human haploid genomes differed at a rate of 1 bp per 1250 on average, but there was marked heterogeneity in the level of polymorphism across the genome. Less than 1% of all SNPs resulted in variation in proteins, but the task of determining which SNPs have functional consequences remains an open challenge.

Decoding of the DNA that constitutes the human genome has been widely anticipated for the contribution it will make toward un-

derstanding human evolution, the causation of disease, and the interplay between the environment and heredity in defining the human condition. A project with the goal of determining the complete nucleotide sequence of the human genome was first formally proposed in 1985 (1). In subsequent years, the idea met with mixed reactions in the scientific community (2). However, in 1990, the Human Genome Project (HGP) was officially initiated in the United States under the direction of the National Institutes of Health and the U.S. Department of Energy with a 15-year, \$3 billion plan for completing the genome sequence. In 1998 we announced our intention to build a unique genome-sequencing facility, to determine the sequence of the human genome over a 3-year period. Here we report the penultimate milestone along the path toward that goal, a nearly complete sequence of the euchromatic portion of the human genome. The sequencing was performed by a whole-genome random shotgun method with subsequent assembly of the sequenced segments.

The modern history of DNA sequencing began in 1977, when Sanger reported his method for determining the order of nucleotides of

DNA using chain-terminating nucleotide analogs (3). In the same year, the first human gene was isolated and sequenced (4). In 1986, Hood and co-workers (5) described an improvement in the Sanger sequencing method that included attaching fluorescent dyes to the nucleotides, which permitted them to be sequentially read by a computer. The first automated DNA sequencer, developed by Applied Biosystems in California in 1987, was shown to be successful when the sequences of two genes were obtained with this new technology (6). From early sequencing of human genomic regions (7), it became clear that cDNA sequences (which are reverse-transcribed from RNA) would be essential to annotate and validate gene predictions in the human genome. These studies were the basis in part for the development of the expressed sequence tag (EST) method of gene identification (8), which is a random selection, very high throughput sequencing approach to characterize cDNA libraries. The EST method led to the rapid discovery and mapping of human genes (9). The increasing numbers of human EST sequences necessitated the development of new computer algorithms to analyze large amounts of sequence data, and in 1993 at The Institute for Genomic Research (TIGR), an algorithm was developed that permitted assembly and analysis of hundreds of thousands of ESTs. This algorithm permitted characterization and annotation of human genes on the basis of 30,000 EST assemblies (10).

The complete 49-kbp bacteriophage lambda genome sequence was determined by a shotgun restriction digest method in 1982 (11). When considering methods for sequencing the smallpox virus genome in 1991 (12), a whole-genome shotgun sequencing method was discussed and subsequently rejected owing to the lack of appropriate software tools for genome assembly. However, in 1994, when a microbial genome-sequencing project was contemplated at TIGR, a whole-genome shotgun sequencing approach was considered possible with the TIGR EST assembly algorithm. In 1995, the 1.8-Mbp *Haemophilus influenzae* genome was completed by a whole-genome shotgun sequencing method (13). The experience with several subsequent genome-sequencing efforts established the broad applicability of this approach (14, 15).

A key feature of the sequencing approach used for these megabase-size and larger genomes was the use of paired-end sequences (also called mate pairs), derived from subclone libraries with distinct insert sizes and cloning characteristics. Paired-end sequences are sequences 500 to 600 bp in length from both ends of double-stranded DNA clones of prescribed lengths. The success of using end sequences from long segments (18 to 20 kbp) of DNA cloned into bacteriophage lambda in assembly of the microbial genomes led to the suggestion (16) of an approach to simulta-

¹Celera Genomics, 45 West Gude Drive, Rockville, MD 20850, USA. ²GenetixXpress, 78 Pacific Road, Palm Beach, Sydney 2108, Australia. ³Berkeley *Drosophila* Genome Project, University of California, Berkeley, CA 94720, USA. ⁴Department of Biology, Penn State University, 208 Mueller Lab, University Park, PA 16802, USA. ⁵Department of Genetics, Case Western Reserve University School of Medicine, BRB-630, 10900 Euclid Avenue, Cleveland, OH 44106, USA. ⁶Johns Hopkins University School of Medicine, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287-4922, USA. ⁷Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA. ⁸New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA. ⁹Division of Biology, 147-75, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA. ¹⁰Yale University School of Medicine, 333 Cedar Street, P.O. Box 208000, New Haven, CT 06520-8000, USA. ¹¹Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA. ¹²The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA. ¹³Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, 52900 Israel. ¹⁴Grup de Recerca en Informàtica Mèdica, Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, 08003-Barcelona, Catalonia, Spain.

*To whom correspondence should be addressed. E-mail: humangenome@celera.com

neously map and sequence the human genome by means of end sequences from 150-kbp bacterial artificial chromosomes (BACs) (17, 18). The end sequences spanned by known distances provide long-range continuity across the genome. A modification of the BAC end-sequencing (BES) method was applied successfully to complete chromosome 2 from the *Arabidopsis thaliana* genome (19).

In 1997, Weber and Myers (20) proposed whole-genome shotgun sequencing of the human genome. Their proposal was not well received (21). However, by early 1998, as less than 5% of the genome had been sequenced, it was clear that the rate of progress in human genome sequencing worldwide was very slow (22), and the prospects for finishing the genome by the 2005 goal were uncertain.

In early 1998, PE Biosystems (now Applied Biosystems) developed an automated, high-throughput capillary DNA sequencer, subsequently called the ABI PRISM 3700 DNA Analyzer. Discussions between PE Biosystems and TIGR scientists resulted in a plan to undertake the sequencing of the human genome with the 3700 DNA Analyzer and the whole-genome shotgun sequencing techniques developed at TIGR (23). Many of the principles of operation of a genome-sequencing facility were established in the TIGR facility (24). However, the facility envisioned for Celera would have a capacity roughly 50 times that of TIGR, and thus new developments were required for sample preparation and tracking and for whole-genome assembly. Some argued that the required 150-fold scale-up from the *H. influenzae* genome to the human genome with its complex repeat sequences was not feasible (25). The *Drosophila melanogaster* genome was thus chosen as a test case for whole-genome assembly on a large and complex eukaryotic genome. In collaboration with Gerald Rubin and the Berkeley *Drosophila* Genome Project, the nucleotide sequence of the 120-Mbp euchromatic portion of the *Drosophila* genome was determined over a 1-year period (26–28). The *Drosophila* genome-sequencing effort resulted in two key findings: (i) that the assembly algorithms could generate chromosome assemblies with highly accurate order and orientation with substantially less than 10-fold coverage, and (ii) that undertaking multiple interim assemblies in place of one comprehensive final assembly was not of value.

These findings, together with the dramatic changes in the public genome effort subsequent to the formation of Celera (29), led to a modified whole-genome shotgun sequencing approach to the human genome. We initially proposed to do 10-fold sequence coverage of the genome over a 3-year period and to make interim assembled sequence data available quarterly. The modifications included a plan to perform random shotgun sequencing to ~5-fold

coverage and to use the unordered and unoriented BAC sequence fragments and subassemblies published in GenBank by the publicly funded genome effort (30) to accelerate the project. We also abandoned the quarterly announcements in the absence of interim assemblies to report.

Although this strategy provided a reasonable result very early that was consistent with a whole-genome shotgun assembly with eight-fold coverage, the human genome sequence is not as finished as the *Drosophila* genome was with an effective 13-fold coverage. However, it became clear that even with this reduced coverage strategy, Celera could generate an accurately ordered and oriented scaffold sequence of the human genome in less than 1 year. Human genome sequencing was initiated 8 September 1999 and completed 17 June 2000. The first assembly was completed 25 June 2000, and the assembly reported here was completed 1 October 2000. Here we describe the whole-genome random shotgun sequencing effort applied to the human genome. We developed two different assembly approaches for assembling the ~3 billion bp that make up the 23 pairs of chromosomes of the *Homo sapiens* genome. Any GenBank-derived data were shredded to remove potential bias to the final sequence from chimeric clones, foreign DNA contamination, or misassembled contigs. Insofar as a correctly and accurately assembled genome sequence with faithful order and orientation of contigs is essential for an accurate analysis of the human genetic code, we have devoted a considerable portion of this manuscript to the documentation of the quality of our reconstruction of the genome. We also describe our preliminary analysis of the human genetic code on the basis of computational methods. Figure 1 (see fold-out chart associated with this issue; files for each chromosome can be found in Web fig. 1 on Science Online at www.sciencemag.org/cgi/content/full/291/5507/1304/DC1) provides a graphical overview of the genome and the features encoded in it. The detailed manual curation and interpretation of the genome are just beginning.

To aid the reader in locating specific analytical sections, we have divided the paper into seven broad sections. A summary of the major results appears at the beginning of each section.

- 1 Sources of DNA and Sequencing Methods
- 2 Genome Assembly Strategy and Characterization
- 3 Gene Prediction and Annotation
- 4 Genome Structure
- 5 Genome Evolution
- 6 A Genome-Wide Examination of Sequence Variations
- 7 An Overview of the Predicted Protein-Coding Genes in the Human Genome
- 8 Conclusions

1 Sources of DNA and Sequencing Methods

Summary. This section discusses the rationale and ethical rules governing donor selection to ensure ethnic and gender diversity along with the methodologies for DNA extraction and library construction. The plasmid library construction is the first critical step in shotgun sequencing. If the DNA libraries are not uniform in size, nonchimeric, and do not randomly represent the genome, then the subsequent steps cannot accurately reconstruct the genome sequence. We used automated high-throughput DNA sequencing and the computational infrastructure to enable efficient tracking of enormous amounts of sequence information (27.3 million sequence reads; 14.9 billion bp of sequence). Sequencing and tracking from both ends of plasmid clones from 2-, 10-, and 50-kbp libraries were essential to the computational reconstruction of the genome. Our evidence indicates that the accurate pairing rate of end sequences was greater than 98%.

Various policies of the United States and the World Medical Association, specifically the Declaration of Helsinki, offer recommendations for conducting experiments with human subjects. We convened an Institutional Review Board (IRB) (31) that helped us establish the protocol for obtaining and using human DNA and the informed consent process used to enroll research volunteers for the DNA-sequencing studies reported here. We adopted several steps and procedures to protect the privacy rights and confidentiality of the research subjects (donors). These included a two-stage consent process, a secure random alphanumeric coding system for specimens and records, circumscribed contact with the subjects by researchers, and options for off-site contact of donors. In addition, Celera applied for and received a Certificate of Confidentiality from the Department of Health and Human Services. This Certificate authorized Celera to protect the privacy of the individuals who volunteered to be donors as provided in Section 301(d) of the Public Health Service Act 42 U.S.C. 241(d).

Celera and the IRB believed that the initial version of a completed human genome should be a composite derived from multiple donors of diverse ethnic backgrounds. Prospective donors were asked, on a voluntary basis, to self-designate an ethnogeographic category (e.g., African-American, Chinese, Hispanic, Caucasian, etc.). We enrolled 21 donors (32).

Three basic items of information from each donor were recorded and linked by confidential code to the donated sample: age, sex, and self-designated ethnogeographic group. From females, ~130 ml of whole, heparinized blood was collected. From males, ~130 ml of whole, heparinized blood was

collected, as well as five specimens of semen, collected over a 6-week period. Permanent lymphoblastoid cell lines were created by Epstein-Barr virus immortalization. DNA from five subjects was selected for genomic DNA sequencing: two males and three females—one African-American, one Asian-Chinese, one Hispanic-Mexican, and two Caucasians (see Web fig. 2 on *Science Online* at www.sciencemag.org/cgi/content/291/5507/1304/DC1). The decision of whose DNA to sequence was based on a complex mix of factors, including the goal of achieving diversity as well as technical issues such as the quality of the DNA libraries and availability of immortalized cell lines.

1.1 Library construction and sequencing

Central to the whole-genome shotgun sequencing process is preparation of high-quality plasmid libraries in a variety of insert sizes so that pairs of sequence reads (mates) are obtained, one read from both ends of each plasmid insert. High-quality libraries have an equal representation of all parts of the genome, a small number of clones without inserts, and no contamination from such sources as the mitochondrial genome and *Escherichia coli* genomic DNA. DNA from each donor was used to construct plasmid libraries in one or more of three size classes: 2 kbp, 10 kbp, and 50 kbp (Table 1) (33).

In designing the DNA-sequencing process, we focused on developing a simple system that could be implemented in a robust and reproducible manner and monitored effectively (Fig. 2) (34).

Current sequencing protocols are based on

the dideoxy sequencing method (35), which typically yields only 500 to 750 bp of sequence per reaction. This limitation on read length has made monumental gains in throughput a prerequisite for the analysis of large eukaryotic genomes. We accomplished this at the Celera facility, which occupies about 30,000 square feet of laboratory space and produces sequence data continuously at a rate of 175,000 total reads per day. The DNA-sequencing facility is supported by a high-performance computational facility (36).

The process for DNA sequencing was modular by design and automated. Intermodule sample backlogs allowed four principal modules to operate independently: (i) library transformation, plating, and colony picking; (ii) DNA template preparation; (iii) dideoxy sequencing reaction set-up and purification; and (iv) sequence determination with the ABI PRISM 3700 DNA Analyzer. Because the inputs and outputs of each module have been carefully matched and sample backlogs are continuously managed, sequencing has proceeded without a single day's interruption since the initiation of the *Drosophila* project in May 1999. The ABI 3700 is a fully automated capillary array sequencer and as such can be operated with a minimal amount of hands-on time, currently estimated at about 15 min per day. The capillary system also facilitates correct associations of sequencing traces with samples through the elimination of manual sample loading and lane-tracking errors associated with slab gels. About 65 production staff were hired and trained, and were rotated on a regular basis

through the four production modules. A central laboratory information management system (LIMS) tracked all sample plates by unique bar code identifiers. The facility was supported by a quality control team that performed raw material and in-process testing and a quality assurance group with responsibilities including document control, validation, and auditing of the facility. Critical to the success of the scale-up was the validation of all software and instrumentation before implementation, and production-scale testing of any process changes.

1.2 Trace processing

An automated trace-processing pipeline has been developed to process each sequence file (37). After quality and vector trimming, the average trimmed sequence length was 543 bp, and the sequencing accuracy was exponentially distributed with a mean of 99.5% and with less than 1 in 1000 reads being less than 98% accurate (26). Each trimmed sequence was screened for matches to contaminants including sequences of vector alone, *E. coli* genomic DNA, and human mitochondrial DNA. The entire read for any sequence with a significant match to a contaminant was discarded. A total of 713 reads matched *E. coli* genomic DNA and 2114 reads matched the human mitochondrial genome.

1.3 Quality assessment and control

The importance of the base-pair level accuracy of the sequence data increases as the size and repetitive nature of the genome to be sequenced increases. Each sequence read must be placed uniquely in the ge-

Table 1. Celera-generated data input into assembly.

	Individual	Number of reads for different insert libraries				Total number of base pairs
		2 kbp	10 kbp	50 kbp	Total	
No. of sequencing reads	A	0	0	2,767,357	2,767,357	1,502,674,851
	B	11,736,757	7,467,755	66,930	19,271,442	10,464,393,006
	C	853,819	881,290	0	1,735,109	942,164,187
	D	952,523	1,046,815	0	1,999,338	1,085,640,534
	F	0	1,498,607	0	1,498,607	813,743,601
	Total	13,543,099	10,894,467	2,834,287	27,271,853	14,808,616,179
Fold sequence coverage (2.9-Gb genome)	A	0	0	0.52	0.52	
	B	2.20	1.40	0.01	3.61	
	C	0.16	1.17	0	0.32	
	D	0.18	0.20	0	0.37	
	F	0	0.28	0	0.28	
	Total	2.54	2.04	0.53	5.11	
Fold clone coverage	A	0	0	18.39	18.39	
	B	2.96	11.26	0.44	14.67	
	C	0.22	1.33	0	1.54	
	D	0.24	1.58	0	1.82	
	F	0	2.26	0	2.26	
	Total	3.42	16.43	18.84	38.68	
Insert size* (mean)	Average	1,951 bp	10,800 bp	50,715 bp		
Insert size* (SD)	Average	6.10%	8.10%	14.90%		
% Mates†	Average	74.50	80.80	75.60		

*Insert size and SD are calculated from assembly of mates on contigs. †% Mates is based on laboratory tracking of sequencing runs.

THE HUMAN GENOME

nome, and even a modest error rate can reduce the effectiveness of assembly. In addition, maintaining the validity of mate-pair information is absolutely critical for the algorithms described below. Procedural controls were established for maintaining the validity of sequence mate-pairs as sequencing reactions proceeded through the process, including strict rules built into the LIMS. The accuracy of sequence data produced by the Celera process was validated in the course of the *Drosophila* genome project (26). By collecting data for the

entire human genome in a single facility, we were able to ensure uniform quality standards and the cost advantages associated with automation, an economy of scale, and process consistency.

2 Genome Assembly Strategy and Characterization

Summary. We describe in this section the two approaches that we used to assemble the genome. One method involves the computational combination of all sequence reads with shredded data from GenBank to generate an indepen-

dent, nonbiased view of the genome. The second approach involves clustering all of the fragments to a region or chromosome on the basis of mapping information. The clustered data were then shredded and subjected to computational assembly. Both approaches provided essentially the same reconstruction of assembled DNA sequence with proper order and orientation. The second method provided slightly greater sequence coverage (fewer gaps) and was the principal sequence used for the analysis phase. In addition, we document the completeness and correctness of this assembly process

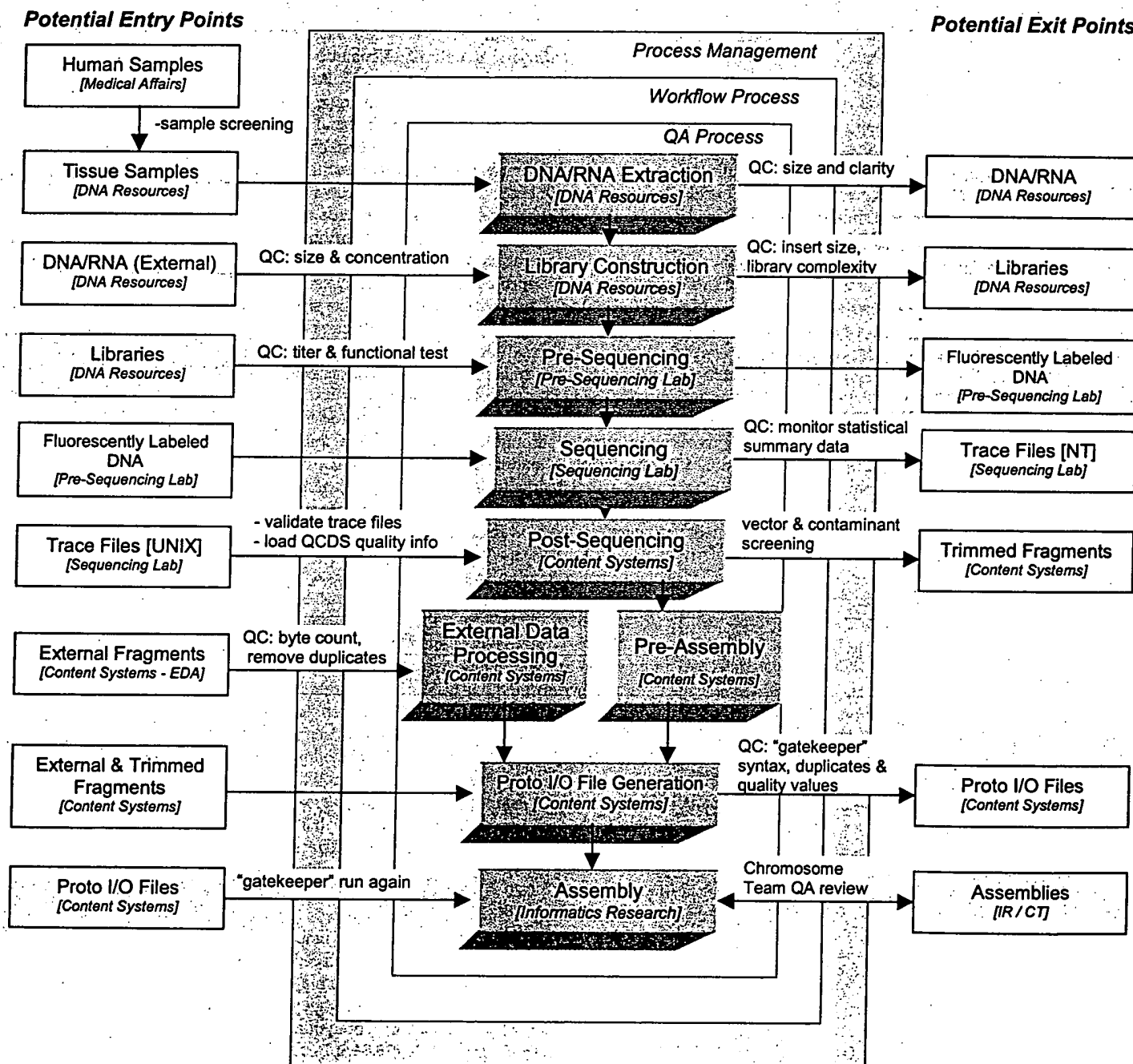


Fig. 2. Flow diagram for sequencing pipeline. Samples are received, selected, and processed in compliance with standard operating procedures, with a focus on quality within and across departments. Each process has defined inputs and outputs with the capability to exchange

samples and data with both internal and external entities according to defined quality guidelines. Manufacturing pipeline processes, products, quality control measures, and responsible parties are indicated and are described further in the text.

and provide a comparison to the public genome sequence, which was reconstructed largely by an independent BAC-by-BAC approach. Our assemblies effectively covered the euchromatic regions of the human chromosomes. More than 90% of the genome was in scaffold assemblies of 100,000 bp or greater, and 25% of the genome was in scaffolds of 10 million bp or larger.

Shotgun sequence assembly is a classic example of an inverse problem: given a set of reads randomly sampled from a target sequence, reconstruct the order and the position of those reads in the target. Genome assembly algorithms developed for *Drosophila* have now been extended to assemble the ~25-fold larger human genome. Celera assemblies consist of a set of contigs that are ordered and oriented into scaffolds that are then mapped to chromosomal locations by using known markers. The contigs consist of a collection of overlapping sequence reads that provide a consensus reconstruction for a contiguous interval of the genome. Mate pairs are a central component of the assembly strategy. They are used to produce scaffolds in which the size of gaps between consecutive contigs is known with reasonable precision. This is accomplished by observing that a pair of reads, one of which is in one contig, and the other of which is in another, implies an orientation and distance between the two contigs (Fig. 3). Finally, our assemblies did not incorporate all reads into the final set of reported scaffolds. This set of unincorporated reads is termed "chaff," and typically consisted of reads from within highly repetitive regions, data from other organisms introduced through various routes as found in many genome projects, and data of poor quality or with untrimmed vector.

2.1 Assembly data sets

We used two independent sets of data for our assemblies. The first was a random shotgun data set of 27.27 million reads of average length 543 bp produced at Celera. This consisted largely of mate-pair reads from 16 libraries constructed from DNA samples taken from five different donors. Libraries with insert sizes of 2, 10, and 50 kbp were used. By looking at how mate pairs from a library were positioned in known sequenced stretches of the genome, we were able to characterize the range of insert sizes in each library and determine a mean and standard deviation. Table 1 details the number of reads, sequencing coverage, and clone coverage achieved by the data set. The clone coverage is the coverage of the genome in cloned DNA, considering the entire insert of each clone that has sequence from both ends. The clone coverage provides a measure of the amount of physical DNA coverage of the genome. Assuming a genome size of 2.9 Gbp, the Celera trimmed sequences gave a 5.1 \times coverage of the genome, and clone coverage was 3.42 \times , 16.40 \times , and 18.84 \times for the 2-, 10-, and 50-kbp libraries, respectively, for a total of 38.7 \times clone coverage.

The second data set was from the publicly funded Human Genome Project (PFP) and is primarily derived from BAC clones (30). The BAC data input to the assemblies came from a download of GenBank on 1 September 2000 (Table 2) totaling 4443.3 Mbp of sequence. The data for each BAC is deposited at one of four levels of completion. Phase 0 data are a set of generally unassembled sequencing reads from a very light shotgun of the BAC, typically less than 1 \times . Phase 1 data are unordered assemblies of contigs, which we call BAC contigs or bactigs. Phase 2 data are ordered assemblies of bactigs. Phase 3 data are complete BAC

sequences. In the past 2 years the PFP has focused on a product of lower quality and completeness, but on a faster time-course, by concentrating on the production of Phase 1 data from a 3 \times to 4 \times light-shotgun of each BAC clone.

We screened the bactig sequences for contaminants by using the BLAST algorithm against three data sets: (i) vector sequences in Univec core (38), filtered for a 25-bp match at 98% sequence identity at the ends of the sequence and a 30-bp match internal to the sequence; (ii) the nonhuman portion of the High Throughput Genomic (HTG) Sequences division of GenBank (39), filtered at 200 bp at 98%; and (iii) the non-redundant nucleotide sequences from GenBank without primate and human virus entries, filtered at 200 bp at 98%. Whenever 25 bp or more of vector was found within 50 bp of the end of a contig, the tip up to the matching vector was excised. Under these criteria we removed 2.6 Mbp of possible contaminant and vector from the Phase 3 data, 61.0 Mbp from the Phase 1 and 2 data, and 16.1 Mbp from the Phase 0 data (Table 2). This left us with a total of 4363.7 Mbp of PFP sequence data 20% finished, 75% rough-draft (Phase 1 and 2), and 5% single sequencing reads (Phase 0). An additional 104,018 BAC end-sequence mate pairs were also downloaded and included in the data sets for both assembly processes (18).

2.2 Assembly strategies

Two different approaches to assembly were pursued. The first was a whole-genome assembly process that used Celera data and the PFP data in the form of additional synthetic shotgun data, and the second was a compartmentalized assembly process that first partitioned the Celera and PFP data into sets localized to large chromosomal segments and then performed ab initio shotgun assembly on each set. Figure 4 gives a schematic of the overall process flow.

For the whole-genome assembly, the PFP data was first disassembled or "shredded" into a synthetic shotgun data set of 550-bp reads that form a perfect 2 \times covering of the bactigs. This resulted in 16.05 million "faux" reads that were sufficient to cover the genome 2.96 \times because of redundancy in the BAC data set, without incorporating the biases inherent in the PFP assembly process. The combined data set of 43.32 million reads (8 \times), and all associated mate-pair information, were then subjected to our whole-genome assembly algorithm to produce a reconstruction of the genome. Neither the location of a BAC in the genome nor its assembly of bactigs was used in this process. Bactigs were shredded into reads because we found strong evidence that 2.13% of them were misassembled (40). Furthermore, BAC location

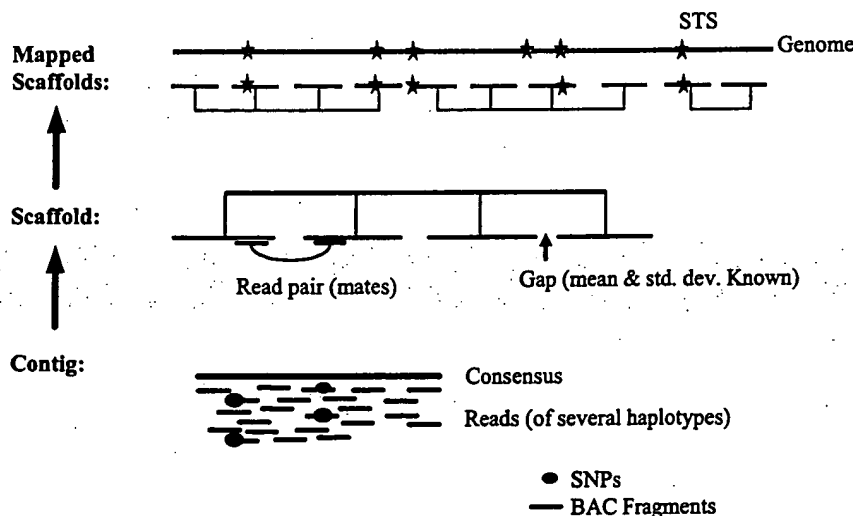


Fig. 3. Anatomy of whole-genome assembly. Overlapping shredded bactig fragments (red lines) and internally derived reads from five different individuals (black lines) are combined to produce a contig and a consensus sequence (green line). Contigs are connected into scaffolds (red) by using mate pair information. Scaffolds are then mapped to the genome (gray line) with STS (blue star) physical map information.

THE HUMAN GENOME

information was ignored because some BACs were not correctly placed on the PFP physical map and because we found strong evidence that at least 2.2% of the BACs contained sequence data that were not part of the given BAC (41), possibly as a result of sample-tracking errors

Table 2. GenBank data input into assembly.

Center	Statistics	Completion phase sequence		
		0	1 and 2	3
Whitehead Institute/ MIT Center for Genome Research, USA	Number of accession records	2,825	6,533	363
	Number of contigs	243,786	138,023	363
	Total base pairs	194,490,158	1,083,848,245	48,829,358
	Total vector masked (bp)	1,553,597	875,618	2,202
	Total contaminant masked (bp)	13,654,482	4,417,055	98,028
	Average contig length (bp)	798	7,853	134,516
Washington University, USA	Number of accession records	19	3,232	1,300
	Number of contigs	2,127	61,812	1,300
	Total base pairs	1,195,732	561,171,788	164,214,395
	Total vector masked (bp)	21,604	270,942	8,287
	Total contaminant masked (bp)	22,469	1,476,141	469,487
	Average contig length (bp)	562	9,079	126,319
Baylor College of Medicine, USA	Number of accession records	0	1,626	363
	Number of contigs	0	44,861	363
	Total base pairs	0	265,547,066	49,017,104
	Total vector masked (bp)	0	218,769	4,960
	Total contaminant masked (bp)	0	1,784,700	485,137
	Average contig length (bp)	0	5,919	135,033
Production Sequencing Facility, DOE Joint Genome Institute, USA	Number of accession records	135	2,043	754
	Number of contigs	7,052	34,938	754
	Total base pairs	8,680,214	294,249,631	60,975,328
	Total vector masked (bp)	22,644	162,651	7,274
	Total contaminant masked (bp)	665,818	4,642,372	118,387
	Average contig length (bp)	1,231	8,422	80,867
The Institute of Physical and Chemical Research (RIKEN), Japan	Number of accession records	0	1,149	300
	Number of contigs	0	25,772	300
	Total base pairs	0	182,812,275	20,093,926
	Total vector masked (bp)	0	203,792	2,371
	Total contaminant masked (bp)	0	308,426	27,781
	Average contig length (bp)	0	7,093	66,978
Sanger Centre, UK	Number of accession records	0	4,538	2,599
	Number of contigs	0	74,324	2,599
	Total base pairs	0	689,059,692	246,118,000
	Total vector masked (bp)	0	427,326	25,054
	Total contaminant masked (bp)	0	2,066,305	374,561
	Average contig length (bp)	0	9,271	94,697
Others*	Number of accession records	42	1,894	3,458
	Number of contigs	5,978	29,898	3,458
	Total base pairs	5,564,879	283,358,877	246,474,157
	Total vector masked (bp)	57,448	279,477	32,136
	Total contaminant masked (bp)	575,366	1,616,665	1,791,849
	Average contig length (bp)	931	9,478	71,277
All centers combined†	Number of accession records	3,021	21,015	9,137
	Number of contigs	258,943	409,628	9,137
	Total base pairs	209,930,983	3,360,047,574	835,722,268
	Total vector masked (bp)	1,655,293	2,438,575	82,284
	Total contaminant masked (bp)	14,918,135	16,311,664	3,365,230
	Average contig length (bp)	811	8,203	91,466

*Other centers contributing at least 0.1% of the sequence include: Chinese National Human Genome Center; Genomanalyse Gesellschaft fuer Biotechnologische Forschung mbH; Genome Therapeutics Corporation; GENOSCOPE; Chinese Academy of Sciences; Institute of Molecular Biotechnology; Keio University School of Medicine; Lawrence Livermore National Laboratory; Cold Spring Harbor Laboratory; Los Alamos National Laboratory; Max-Planck Institut fuer Molekulare Genetik; Japan Science and Technology Corporation; Stanford University; The Institute for Genomic Research; The Institute of Physical and Chemical Research, Gene Bank; The University of Oklahoma; University of Texas Southwestern Medical Center, University of Washington. †The 4,405,700,825 bases contributed by all centers were shredded into faux reads resulting in 2.96X coverage of the genome.

(see below). In short, we performed a true, ab initio whole-genome assembly in which we took the expedient of deriving additional sequence coverage, but not mate pairs, assembled bactigs, or genome locality, from some externally generated data.

In the compartmentalized shotgun assembly (CSA), Celera and PFP data were partitioned into the largest possible chromosomal segments or "components" that could be determined with confidence, and then shotgun assembly was applied to each partitioned subset wherein the bactig data were again shredded into faux reads to ensure an independent ab initio assembly of the component. By subsetting the data in this way, the overall computational effort was reduced and the effect of interchromosomal duplications was ameliorated. This also resulted in a reconstruction of the genome that was relatively independent of the whole-genome assembly results so that the two assemblies could be compared for consistency. The quality of the partitioning into components was crucial so that different genome regions were not mixed together. We constructed components from (i) the longest scaffolds of the sequence from each BAC and (ii) assembled scaffolds of data unique to Celera's data set. The BAC assemblies were obtained by a combining assembler that used the bactigs and the 5X Celera data mapped to those bactigs as input. This effort was undertaken as an interim step solely because the more accurate and complete the scaffold for a given sequence stretch, the more accurately one can tile these scaffolds into contiguous components on the basis of sequence overlap and mate-pair information. We further visually inspected and curated the scaffold tiling of the components to further increase its accuracy. For the final CSA assembly, all but the partitioning was ignored, and an independent, ab initio reconstruction of the sequence in each component was obtained by applying our whole-genome assembly algorithm to the partitioned, relevant Celera data and the shredded, faux reads of the partitioned, relevant bactig data.

2.3 Whole-genome assembly

The algorithms used for whole-genome assembly (WGA) of the human genome were enhancements to those used to produce the sequence of the *Drosophila* genome reported in detail in (28).

The WGA assembler consists of a pipeline composed of five principal stages: Screener, Overlapper, Unittigger, Scaffolder, and Repeat Resolver, respectively. The Screener finds and marks all microsatellite repeats with less than a 6-bp element, and screens out all known interspersed repeat elements, including Alu, Line, and ribosomal DNA. Marked regions get searched for overlaps, whereas screened regions do not get searched, but can be part of an overlap that involves unscreened matching segments.

The Overlapper compares every read against every other read in search of complete end-to-end overlaps of at least 40 bp and with no more than 6% differences in the match. Because all data are scrupulously vector-trimmed, the Overlapper can insist on complete overlap matches. Computing the set of all overlaps took roughly 10,000 CPU hours with a suite of four-processor Alpha SMPs with 4 gigabytes of RAM. This took 4 to 5 days in elapsed time with 40 such machines operating in parallel.

Every overlap computed above is statistically a 1-in- 10^{17} event and thus not a coincidental event. What makes assembly combinatorially difficult is that while many overlaps are actually sampled from overlapping regions of the genome, and thus imply that the sequence reads should be assembled together, even more overlaps are actually from two distinct copies of a low-copy repeated element not screened above, thus constituting an error if put together. We call the former "true overlaps" and the latter "repeat-induced overlaps." The assembler must avoid choosing repeat-induced overlaps, especially early in the process.

We achieve this objective in the Unitigger. We first find all assemblies of reads that appear to be uncontested with respect to all other reads. We call the contigs formed from these subassemblies unitigs (for uniquely assembled contigs). Formally, these unitigs are the uncontested interval subgraphs of the graph of all overlaps (42). Unfortunately, although empirically many of these assemblies are correct (and thus involve only true overlaps), some are in fact collections of reads from several copies of a repetitive element that have been overcollapsed into a single subassembly. However, the overcollapsed unitigs are easily identified because their average coverage depth is too high to be consistent with the overall level of sequence coverage. We developed a simple statistical discriminator that gives the logarithm of the odds ratio that a unitig is composed of unique DNA or of a repeat consisting of two or more copies. The discriminator, set to a sufficiently stringent threshold, identifies a subset of the unitigs that we are certain are correct. In addition, a second, less stringent threshold identifies a subset of remaining unitigs very likely to be correctly assembled, of which we select those that will consistently scaffold (see below), and thus are again almost certain to be correct. We call the union of these two sets U-unitigs. Empirically, we found from a 6× simulated shotgun of human chromosome 22 that we get U-unitigs covering 98% of the stretches of unique DNA that are >2 kbp long. We are further able to identify the boundary of the start of a repetitive element at the ends of a U-unitig and leverage this so that U-unitigs span more than 93% of all

uniquely interspersed Alu elements and other 100-to 400-bp repetitive segments.

The result of running the Unitigger was thus a set of correctly assembled subcontigs covering an estimated 73.6% of the human genome. The Scaffolders then proceeded to use mate-pair information to link these together into scaffolds. When there are two or more mate pairs that imply that a given pair of U-unitigs are at a certain distance and orientation with respect to each other, the probability of this being wrong is again roughly 1 in 10^{10} , assuming that mate pairs are false less than 2% of the time. Thus, one can with high confidence link together all U-unitigs that are linked by at least two 2- or 10-kbp mate pairs producing intermediate-sized scaffolds that are then recursively linked together by confirming 50-kbp mate pairs and BAC end sequences. This process yielded scaffolds that are on the order of megabase pairs in size with gaps between their contigs that generally correspond to repetitive elements and occasionally to small sequencing gaps. These scaffolds reconstruct the majority of the unique sequence within a genome.

For the *Drosophila* assembly, we engaged in a three-stage repeat resolution strategy where each stage was progressively more

aggressive and thus more likely to make a mistake. For the human assembly, we continued to use the first "Rocks" substage where all unitigs with a good, but not definitive, discriminator score are placed in a scaffold gap. This was done with the condition that two or more mate pairs with one of their reads already in the scaffold unambiguously place the unitig in the given gap. We estimate the probability of inserting a unitig into an incorrect gap with this strategy to be less than 10^{-7} based on a probabilistic analysis.

We revised the ensuing "Stones" substage of the human assembly, making it more like the mechanism suggested in our earlier work (43). For each gap, every read R that is placed in the gap by virtue of its mated pair M being in a contig of the scaffold and implying R's placement is collected. Celera's mate-pairing information is correct more than 99% of the time. Thus, almost every, but not all, of the reads in the set belong in the gap, and when a read does not belong it rarely agrees with the remainder of the reads. Therefore, we simply assemble this set of reads within the gap, eliminating any reads that conflict with the assembly. This operation proved much more reliable than the one it replaced for the *Drosophila* assembly; in the assembly of a simulated shotgun data set of human chromo-

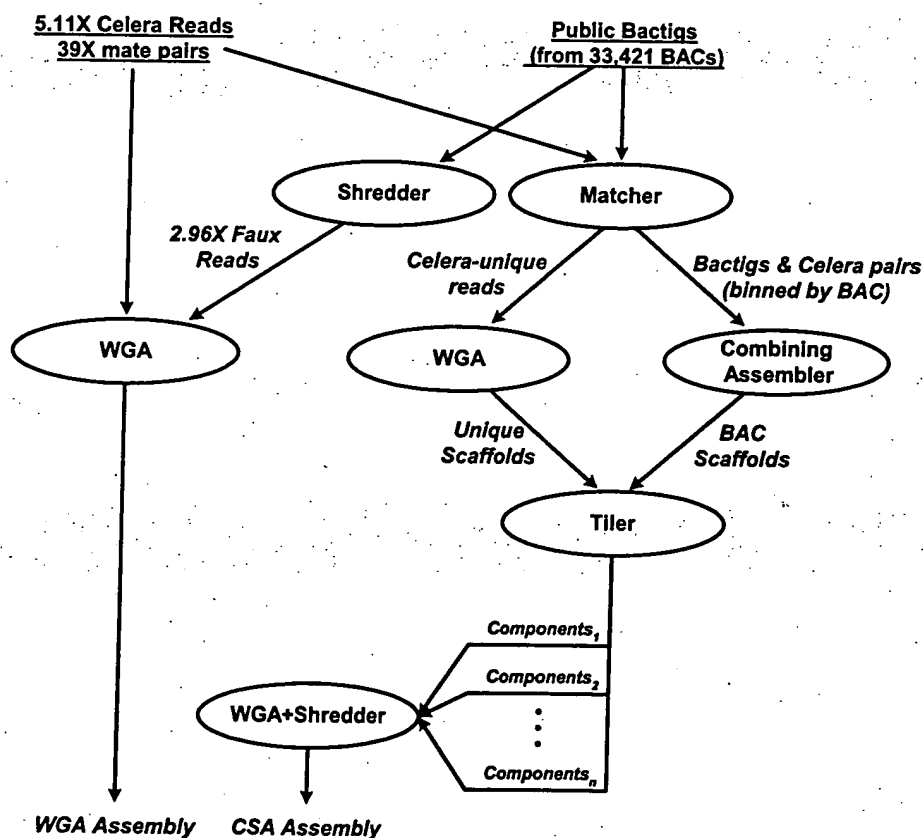


Fig. 4. Architecture of Celera's two-pronged assembly strategy. Each oval denotes a computation process performing the function indicated by its label, with the labels on arcs between ovals describing the nature of the objects produced and/or consumed by a process. This figure summarizes the discussion in the text that defines the terms and phrases used.

some 22, all stones were placed correctly.

The final method of resolving gaps is to fill them with assembled BAC data that cover the gap. We call this external gap "walking." We did not include the very aggressive "Pebbles" substage described in our *Drosophila* work, which made enough mistakes so as to produce repeat reconstructions for long interspersed elements whose quality was only 99.62% correct. We decided that for the human genome it was philosophically better not to introduce a step that was certain to produce less than 99.99% accuracy. The cost was a somewhat larger number of gaps of somewhat larger size.

At the final stage of the assembly process, and also at several intermediate points, a consensus sequence of every contig is produced. Our algorithm is driven by the principle of maximum parsimony, with quality-value-weighted measures for evaluating each base. The net effect is a Bayesian estimate of the correct base to report at each position. Consensus generation uses Celera data whenever it is present. In the event that no Celera data cover a given region, the BAC data sequence is used.

A key element of achieving a WGA of the human genome was to parallelize the Overlapper and the central consensus sequence-constructing subroutines. In addition, memory was a real issue—a straightforward application of the software we had built for *Drosophila* would

have required a computer with a 600-gigabyte RAM. By making the Overlapper and Unittigger incremental, we were able to achieve the same computation with a maximum of instantaneous usage of 28 gigabytes of RAM. Moreover, the incremental nature of the first three stages allowed us to continually update the state of this part of the computation as data were delivered and then perform a 7-day run to complete Scaffolding and Repeat Resolution whenever desired. For our assembly operations, the total compute infrastructure consists of 10 four-processor SMPs with 4 gigabytes of memory per cluster (Compaq's ES40, Regatta) and a 16-processor NUMA machine with 64 gigabytes of memory (Compaq's GS160, Wildfire). The total compute for a run of the assembler was roughly 20,000 CPU hours.

The assembly of Celera's data, together with the shredded bactig data, produced a set of scaffolds totaling 2.848 Gbp in span and consisting of 2.586 Gbp of sequence. The chaff, or set of reads not incorporated in the assembly, numbered 11.27 million (26%), which is consistent with our experience for *Drosophila*. More than 84% of the genome was covered by scaffolds >100 kbp long, and these averaged 91% sequence and 9% gaps with a total of 2.297 Gbp of sequence. There were a total of 93,857 gaps among the 1637 scaffolds >100 kbp. The average scaffold size was 1.5 Mbp; the average contig size was 24.06 kbp, and the average gap size was 2.43 kbp, where the dis-

tribution of each was essentially exponential. More than 50% of all gaps were less than 50 bp long, >62% of all gaps were less than 1 kbp long, and no gap was >100 kbp long. Similarly, more than 65% of the sequence is in contigs >30 kbp, more than 31% is in contigs >100 kbp, and the largest contig was 1.22 Mbp long. Table 3 gives detailed summary statistics for the structure of this assembly with a direct comparison to the compartmentalized shotgun assembly.

2.4 Compartmentalized shotgun assembly

In addition to the WGA approach, we pursued a localized assembly approach that was intended to subdivide the genome into segments, each of which could be shotgun assembled individually. We expected that this would help in resolution of large interchromosomal duplications and improve the statistics for calculating U-units. The compartmentalized assembly process involved clustering Celera reads and bactigs into large, multiple megabase regions of the genome, and then running the WGA assembler on the Celera data and shredded, faux reads obtained from the bactig data.

The first phase of the CSA strategy was to separate Celera reads into those that matched the BAC contigs for a particular PFP BAC entry, and those that did not match any public data. Such matches must be guaranteed to

Table 3. Scaffold statistics for whole-genome and compartmentalized shotgun assemblies.

	Scaffold size				
	All	>30 kbp	>100 kbp	>500 kbp	>1000 kbp
Compartmentalized shotgun assembly					
No. of bp in scaffolds (including intrascaffold gaps)	2,905,568,203	2,748,892,430	2,700,489,906	2,489,357,260	2,248,689,128
No. of bp in contigs	2,653,979,733	2,524,251,302	2,491,538,372	2,320,648,201	2,106,521,902
No. of scaffolds	53,591	2,845	1,935	1,060	721
No. of contigs	170,033	112,207	107,199	93,138	82,009
No. of gaps	116,442	109,362	105,264	92,078	81,288
No. of gaps ≤1 kbp	72,091	69,175	67,289	59,915	53,354
Average scaffold size (bp)	54,217	966,219	1,395,602	2,348,450	3,118,848
Average contig size (bp)	15,609	22,496	23,242	24,916	25,686
Average intrascaffold gap size (bp)	2,161	2,054	1,985	1,832	1,749
Largest contig (bp)	1,988,321	1,988,321	1,988,321	1,988,321	1,988,321
% of total contigs	100	95	94	87	79
Whole-genome assembly					
No. of bp in scaffolds (including intrascaffold gaps)	2,847,890,390	2,574,792,618	2,525,334,447	2,328,535,466	2,140,943,032
No. of bp in contigs	2,586,634,108	2,334,343,339	2,297,678,935	2,143,002,184	1,983,305,432
No. of scaffolds	118,968	2,507	1,637	818	554
No. of contigs	221,036	99,189	95,494	84,641	76,285
No. of gaps	102,068	96,682	93,857	83,823	75,731
No. of gaps ≤1 kbp	62,356	60,343	59,156	54,079	49,592
Average scaffold size (bp)	23,938	1,027,041	1,542,660	2,846,620	3,864,518
Average contig size (bp)	11,702	23,534	24,061	25,319	25,999
Average intrascaffold gap size (bp)	2,560	2,487	2,426	2,213	2,082
Largest contig (bp)	1,224,073	1,224,073	1,224,073	1,224,073	1,224,073
% of total contigs	100	90	89	83	77

properly place a Celera read, so all reads were first masked against a library of common repetitive elements, and only matches of at least 40 bp to unmasked portions of the read constituted a hit. Of Celera's 27.27 million reads, 20.76 million matched a bactig and another 0.62 million reads, which did not have any matches, were nonetheless identified as belonging in the region of the bactig's BAC because their mate matched the bactig. Of the remaining reads, 2.92 million were completely screened out and so could not be matched, but the other 2.97 million reads had unmasked sequence totaling 1.189 Gbp that were not found in the GenBank data set. Because the Celera data are $5.11\times$ redundant, we estimate that 240 Mbp of unique Celera sequence is not in the GenBank data set.

In the next step of the CSA process, a combining assembler took the relevant $5\times$ Celera reads and bactigs for a BAC entry, and produced an assembly of the combined data for that locale. These high-quality sequence reconstructions were a transient result whose utility was simply to provide more reliable information for the purposes of their tiling into sets of overlapping and adjacent scaffold sequences in the next step. In outline, the combining assembler first examines the set of matching Celera reads to determine if there are excessive pileups indicative of unscreened repetitive elements. Wherever these occur, reads in the repeat region whose mates have not been mapped to consistent positions are removed. Then all sets of mate pairs that consistently imply the same relative position of two bactigs are bundled into a link and weighted according to the number of mates in the bundle. A "greedy" strategy then attempts to order the bactigs by selecting bundles of mate-pairs in order of their weight. A selected mate-pair bundle can tie together two formative scaffolds. It is incorporated to form a single scaffold only if it is consistent with the majority of links between contigs of the scaffold. Once scaffolding is complete, gaps are filled by the "Stones" strategy described above for the WGA assembler.

The GenBank data for the Phase 1 and 2 BACs consisted of an average of 19.8 bactigs per BAC of average size 8099 bp. Application of the combining assembler resulted in individual Celera BAC assemblies being put together into an average of 1.83 scaffolds (median of 1 scaffold) consisting of an average of 8.57 contigs of average size 18,973 bp. In addition to defining order and orientation of the sequence fragments, there were 57% fewer gaps in the combined result. For Phase 0 data, the average GenBank entry consisted of 91.52 reads of average length 784 bp. Application of the combining assembler resulted in an average of 54.8 scaffolds consisting of an average of 58.1 contigs of average size 873 bp. Basically, some small amount of

assembly took place, but not enough Celera data were matched to truly assemble the $0.5\times$ to $1\times$ data set represented by the typical Phase 0 BACs. The combining assembler was also applied to the Phase 3 BACs for SNP identification, confirmation of assembly, and localization of the Celera reads. The phase 0 data suggest that a combined whole-genome shotgun data set and $1\times$ light-shotgun of BACs will not yield good assembly of BAC regions; at least $3\times$ light-shotgun of each BAC is needed.

The 5.89 million Celera fragments not matching the GenBank data were assembled with our whole-genome assembler. The assembly resulted in a set of scaffolds totaling 442 Mbp in span and consisting of 326 Mbp of sequence. More than 20% of the scaffolds were >5 kbp long, and these averaged 63% sequence and 27% gaps with a total of 302 Mbp of sequence. All scaffolds >5 kbp were forwarded along with all scaffolds produced by the combining assembler to the subsequent tiling phase.

At this stage, we typically had one or two scaffolds for every BAC region constituting at least 95% of the relevant sequence, and a collection of disjoint Celera-unique scaffolds. The next step in developing the genome components was to determine the order and overlap tiling of these BAC and Celera-unique scaffolds across the genome. For this, we used Celera's 50-kbp mate-pairs information, and BAC-end pairs (18) and sequence tagged site (STS) markers (44) to provide long-range guidance and chromosome separation. Given the relatively manageable number of scaffolds, we chose not to produce this tiling in a fully automated manner, but to compute an initial tiling with a good heuristic and then use human curators to resolve discrepancies or missed join opportunities. To this end, we developed a graphical user interface that displayed the graph of tiling overlaps and the evidence for each. A human curator could then explore the implication of mapped STS data, dot-plots of sequence overlap, and a visual display of the mate-pair evidence supporting a given choice. The result of this process was a collection of "components," where each component was a tiled set of BAC and Celera-unique scaffolds that had been curator-approved. The process resulted in 3845 components with an estimated span of 2.922 Gbp.

In order to generate the final CSA, we assembled each component with the WGA algorithm. As was done in the WGA process, the bactig data were shredded into a synthetic $2\times$ shotgun data set in order to give the assembler the freedom to independently assemble the data. By using faux reads rather than bactigs, the assembly algorithm could correct errors in the assembly of bactigs and remove chimeric content in a PFP data entry.

Chimeric or contaminating sequence (from another part of the genome) would not be incorporated into the reassembly of the component because it did not belong there. In effect, the previous steps in the CSA process served only to bring together Celera fragments and PFP data relevant to a large contiguous segment of the genome, wherein we applied the assembler used for WGA to produce an ab initio assembly of the region.

WGA assembly of the components resulted in a set of scaffolds totaling 2.906 Gbp in span and consisting of 2.654 Gbp of sequence. The chaff, or set of reads not incorporated into the assembly, numbered 6.17 million, or 22%. More than 90.0% of the genome was covered by scaffolds spanning >100 kbp long, and these averaged 92.2% sequence and 7.8% gaps with a total of 2.492 Gbp of sequence. There were a total of 105,264 gaps among the 107,199 contigs that belong to the 1940 scaffolds spanning >100 kbp. The average scaffold size was 1.4 Mbp, the average contig size was 23.24 kbp, and the average gap size was 2.0 kbp where each distribution of sizes was exponential. As such, averages tend to be underrepresentative of the majority of the data. Figure 5 shows a histogram of the bases in scaffolds of various size ranges. Consider also that more than 49% of all gaps were <500 bp long, more than 62% of all gaps were <1 kbp, and all gaps are <100 kbp long. Similarly, more than 73% of the sequence is in contigs >30 kbp, more than 49% is in contigs >100 kbp, and the largest contig was 1.99 Mbp long. Table 3 provides summary statistics for the structure of this assembly with a direct comparison to the WGA assembly.

2.5 Comparison of the WGA and CSA scaffolds

Having obtained two assemblies of the human genome via independent computational processes (WGA and CSA), we compared scaffolds from the two assemblies as another means of investigating their completeness, consistency, and contiguity. From each assembly, a set of reference scaffolds containing at least 1000 fragments (Celera sequencing reads or bactig shreds) was obtained; this amounted to 2218 WGA scaffolds and 1717 CSA scaffolds, for a total of 2.087 Gbp and 2.474 Gbp. The sequence of each reference scaffold was compared to the sequence of all scaffolds from the other assembly with which it shared at least 20 fragments or at least 20% of the fragments of the smaller scaffold. For each such comparison, all matches of at least 200 bp with at most 2% mismatch were tabulated.

From this tabulation, we estimated the amount of unique sequence in each assembly in two ways. The first was to determine the number of bases of each assembly that were

not covered by a matching segment in the other assembly. Some 82.5 Mbp of the WGA (3.95%) was not covered by the CSA, whereas 204.5 Mbp (8.26%) of the CSA was not covered by the WGA. This estimate did not require any consistency of the assemblies or any uniqueness of the matching segments. Thus, another analysis was conducted in which matches of less than 1 kbp between a pair of scaffolds were excluded unless they were confirmed by other matches having a consistent order and orientation. This gives some measure of consistent coverage: 1.982 Gbp (95.00%) of the WGA is covered by the CSA, and 2.169 Gbp (87.69%) of the CSA is covered by the WGA by this more stringent measure.

The comparison of WGA to CSA also permitted evaluation of scaffolds for structural inconsistencies. We looked for instances in which a large section of a scaffold from one assembly matched only one scaffold from the other assembly, but failed to match over the full length of the overlap implied by the matching segments. An initial set of candidates was identified automatically, and then each candidate was inspected by hand. From this process, we identified 31 instances in which the assemblies appear to disagree in a nonlocal fashion. These cases are being further evaluated to determine which assembly is in error and why.

In addition, we evaluated local inconsistencies of order or orientation. The following results exclude cases in which one contig in one assembly corresponds to more than one overlapping contig in the other assembly (as long as the order and orientation of the latter agrees with the positions they match in the former). Most of these small rearrangements involved segments on the order of hundreds of base pairs and rarely >1 kbp. We found a total of 295 kbp (0.012%) in the CSA assemblies that were locally inconsistent with the WGA assemblies, whereas 2.108 Mbp (0.11%) in the WGA assembly were inconsistent with the CSA assembly.

The CSA assembly was a few percentage points better in terms of coverage and slightly more consistent than the WGA, because it was in effect performing a few thousand shotgun assemblies of megabase-sized problems, whereas the WGA is performing a shotgun assembly of a gigabase-sized problem. When one considers the increase of two-and-a-half orders of magnitude in problem size, the information loss between the two is remarkably small. Because CSA was logistically easier to deliver and the better of the two results available at the time when downstream analyses needed to be begun, all subsequent analysis was performed on this assembly.

2.6 Mapping scaffolds to the genome

The final step in assembling the genome was to order and orient the scaffolds on the chromosomes. We first grouped scaffolds together on the basis of their order in the components from CSA. These grouped scaffolds were reordered by examining residual mate-pairing data between the scaffolds. We next mapped the scaffold groups onto the chromosome using physical mapping data. This step depends on having reliable high-resolution map information such that each scaffold will overlap multiple markers. There are two genome-wide types of map information available: high-density STS maps and fingerprint maps of BAC clones developed at Washington University (45). Among the genome-wide STS maps, GeneMap99 (GM99) has the most markers and therefore was most useful for mapping scaffolds. The two different mapping approaches are complementary to one another. The fingerprint maps should have better local order because they were built by comparison of overlapping BAC clones. On the other hand, GM99 should have a more reliable long-range order, because the framework markers were derived from well-validated genetic maps. Both types of maps were used as a reference for human curation of the components that were the input to the regional assembly, but they did not determine the order of sequences produced by the assembler.

In order to determine the effectiveness of the fingerprint maps and GM99 for mapping scaffolds, we first examined the reliability of these maps by comparison with large scaffolds. Only 1% of the STS markers on the 10 largest scaffolds (those >9 Mbp) were mapped on a different chromosome on GM99. Two percent of the STS markers disagreed in position by more than five framework bins. However, for the fingerprint maps, a 2% chromosome discrepancy was observed, and on average 23.8% of BAC locations in the scaffold sequence disagreed with fingerprint map placement by more than five BACs. When further examining the source of discrepancy, it was found that most of the discrepancy came from 4 of the 10 scaffolds; indicating this there is variation in the quality of either the map or the scaffolds. All four scaffolds were assembled, as well as the other six, as judged by clone coverage analysis, and showed the same low discrepancy rate to GM99, and thus we concluded that the fingerprint map global order in these cases was not reliable. Smaller scaffolds had a higher discordance rate with GM99 (4.21% of STSs were discordant by more than five framework bins), but a lower discordance rate with the fingerprint maps (11% of BACs disagreed with fingerprint maps by more than five BACs). This observation agrees with the clone coverage analysis (46) that Celera scaffold construction was better supported by long-range mate pairs in larger scaffolds than in small scaffolds.

We created two orderings of Celera scaffolds on the basis of the markers (BAC or STS) on these maps. Where the order of scaffolds agreed between GM99 and the WashU BAC map, we had a high degree of confidence that that order was correct; these scaffolds were termed "anchor scaffolds." Only scaffolds with a low overall discrepancy rate with both maps were considered anchor scaffolds. Scaffolds in GM99 bins were allowed to permute in their order to match WashU ordering, provided they did not violate their framework orders. Orientation of individual scaffolds was determined by the presence of multiple mapped markers with consistent order. Scaffolds with only one marker have insufficient information to assign orientation. We found 70.1% of the genome in anchored scaffolds, more than 99% of which are also oriented (Table 4). Because GM99 is of lower resolution than the WashU map, a number of scaffolds without STS matches could be ordered relative to the anchored scaffolds because they included sequence from the same or adjacent BACs on the WashU map. On the other hand, because of occasional WashU global ordering discrepancies, a number of scaffolds determined to be "unmappable" on the WashU map could be ordered relative to the anchored scaffolds

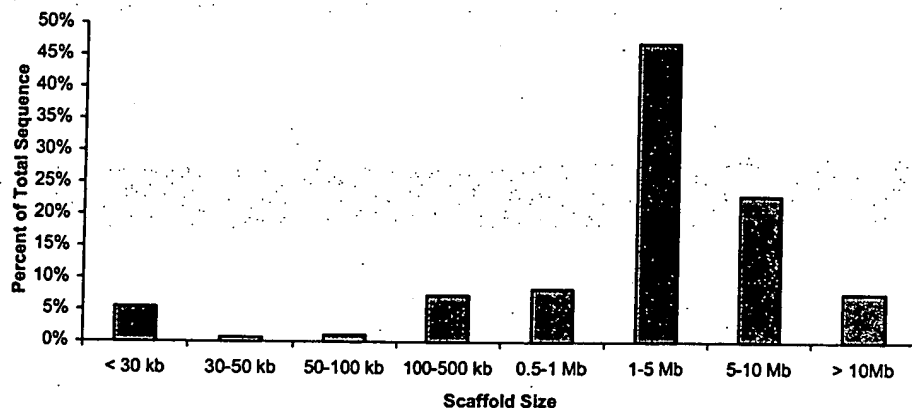


Fig. 5. Distribution of scaffold sizes of the CSA. For each range of scaffold sizes, the percent of total sequence is indicated.

with GM99. These scaffolds were termed "ordered scaffolds." We found that 13.9% of the assembly could be ordered by these additional methods, and thus 84.0% of the genome was ordered unambiguously.

Next, all scaffolds that could be placed, but not ordered, between anchors were assigned to the interval between the anchored scaffolds and were deemed to be "bounded" between them. For example, small scaffolds having STS hits from the same GeneMap bin or hitting the same BAC cannot be ordered relative to each other, but can be assigned a placement boundary relative to other anchored or ordered scaffolds. The remaining scaffolds either had no localization information, conflicting information, or could only be assigned to a generic chromosome location. Using the above approaches, ~98% of the genome was anchored, ordered, or bounded.

Finally, we assigned a location for each scaffold placed on the chromosome by spreading out the scaffolds per chromosome. We assumed that the remaining unmapped scaffolds, constituting 2% of the genome, were distributed evenly across the genome. By dividing the sum of unmapped scaffold lengths with the sum of the number of mapped scaffolds, we arrived at an estimate of interscaffold gap of 1483 bp. This gap was used to separate all the scaffolds on each chromosome and to assign an offset in the chromosome.

During the scaffold-mapping effort, we encountered many problems that resulted in additional quality assessment and validation analysis. At least 978 (3% of 33,173) BACs were believed to have sequence data from more than one location in the genome (47). This is consistent with the *bactig* chimerism analysis reported above in the Assembly Strategies section. These BACs could not be assigned to unique positions within the CSA assembly and thus could not be used for ordering scaffolds. Likewise, it was not always possible to assign STSs to unique locations in the assembly because of genome duplications, repetitive elements, and pseudogenes.

Because of the time required for an exhaustive search for a perfect overlap, CSA generated 21,607 intrascaffold gaps where the mate-pair data suggested that the contigs should overlap, but no overlap was found. These gaps were defined as a fixed 50 bp in length and make up 18.6% of the total 116,442 gaps in the CSA assembly.

We chose not to use the order of exons implied in cDNA or EST data as a way of ordering scaffolds. The rationale for not using this data was that doing so would have biased certain regions of the assembly by rearranging scaffolds to fit the transcript data and made validation of both the assembly and gene definition processes more difficult.

2.7 Assembly and validation analysis

We analyzed the assembly of the genome from the perspectives of completeness (amount of coverage of the genome) and correctness (the structural accuracy of the order and orientation and the consensus sequence of the assembly).

Completeness. Completeness is defined as the percentage of the euchromatic sequence represented in the assembly. This cannot be known with absolute certainty until the euchromatic sequence has been completed. However, it is possible to estimate completeness on the basis of (i) the estimated sizes of intrascaffold gaps; (ii) coverage of the two published chromosomes, 21 and 22 (48, 49); and (iii) analysis of the percentage of an independent set of random sequences (STS markers) contained in the assembly. The whole-genome libraries contain heterochromatic sequence and, although no attempt has been made to assemble it, there may be instances of unique sequence embedded in regions of heterochromatin as were observed in *Drosophila* (50, 51).

The sequences of human chromosomes 21 and 22 have been completed to high quality and published (48, 49). Although this sequence served as input to the assembler, the finished sequence was shredded into a shotgun data set so that the assembler had the opportunity to assemble it differently from the original sequence in the case of structural polymorphisms or assembly errors in the BAC data. In particular, the assembler must be able to resolve repetitive elements at the scale of components (generally multimegabase in size), and so this comparison reveals the level to which the assembler resolves repeats. In certain areas, the assembly structure differs from the published versions of chromosomes 21 and 22 (see below). The consequence of the flexibility to assemble "finished" sequence differently on the basis of Celera data resulted in an assembly with more segments than the chromosome 21 and 22 sequences. We examined the reasons why there are more gaps in the Celera sequence than in chromosomes 21 and 22 and expect that they may be typical of gaps in other regions of the genome. In the Celera assembly, there are 25 scaffolds, each containing at least 10 kb of sequence, that collectively span 94.3% of chromosome 21. Sixty-two scaffolds span 95.7% of chromosome 22. The total length of the gaps remaining in the Celera assembly for these two chromosomes is 3.4 Mbp. These gap sequences were analyzed by RepeatMasker and by searching against the entire genome assembly (52). About 50% of the gap sequence consisted of common repetitive elements identified by RepeatMasker; more than half of the remainder was lower copy number repeat elements.

A more global way of assessing complete-

ness is to measure the content of an independent set of sequence data in the assembly. We compared 48,938 STS markers from Genemap99 (51) to the scaffolds. Because these markers were not used in the assembly processes, they provided a truly independent measure of completeness. ePCR (53) and BLAST (54) were used to locate STSs on the assembled genome. We found 44,524 (91%) of the STSs in the mapped genome. An additional 2648 markers (5.4%) were found by searching the unassembled data or "chaff." We identified 1283 STS markers (2.6%) not found in either Celera sequence or BAC data as of September 2000, raising the possibility that these markers may not be of human origin. If that were the case, the Celera assembled sequence would represent 93.4% of the human genome and the unassembled data 5.5%, for a total of 98.9% coverage. Similarly, we compared CSA against 36,678 TNG radiation hybrid markers (55a) using the same method. We found that 32,371 markers (88%) were located in the mapped CSA scaffolds, with 2055 markers (5.6%) found in the remainder. This gave a 94% coverage of the genome through another genome-wide survey.

Correctness. Correctness is defined as the structural and sequence accuracy of the assembly. Because the source sequences for the Celera data and the GenBank data are from different individuals, we could not directly compare the consensus sequence of the as-

Table 4. Summary of scaffold mapping. Scaffolds were mapped to the genome with different levels of confidence (anchored scaffolds have the highest confidence; unmapped scaffolds have the lowest). Anchored scaffolds were consistently ordered by the WashU BAC map and GM99. Ordered scaffolds were consistently ordered by at least one of the following: the WashU BAC map, GM99, or component tiling path. Bounded scaffolds had order conflicts between at least two of the external maps, but their placements were adjacent to a neighboring anchored or ordered scaffold. Unmapped scaffolds had, at most, a chromosome assignment. The scaffold subcategories are given below each category.

Mapped scaffold category	Number	Length (bp)	% Total length
Anchored	1,526	1,860,676,676	70
Oriented	1,246	1,852,088,645	70
Unoriented	280	8,588,031	0.3
Ordered	2,001	369,235,857	14
Oriented	839	329,633,166	12
Unoriented	1,162	39,602,691	2
Bounded	38,241	368,753,463	14
Oriented	7,453	274,536,424	10
Unoriented	30,788	94,217,039	4
Unmapped	11,823	55,313,737	2
Known chromosome	281	2,505,844	0.1
Unknown chromosome	11,542	52,807,893	2

sembly against other finished sequence for determining sequencing accuracy at the nucleotide level, although this has been done for identifying polymorphisms as described in Section 6. The accuracy of the consensus sequence is at least 99.96% on the basis of a statistical estimate derived from the quality values of the underlying reads.

The structural consistency of the assembly can be measured by mate-pair analysis. In a correct assembly, every mated pair of sequencing reads should be located on the consensus sequence with the correct separation and orientation between the pairs. A pair is termed "valid" when the reads are in the correct orientation and the distance between them is within the mean \pm 3 standard deviations of the distribution of insert sizes of the library from which the pair was sampled. A pair is termed "misoriented" when the reads are not correctly oriented, and is termed "mis-separated" when the distance between the reads is not in the correct range but the reads are correctly oriented. The mean \pm the standard deviation of each library used by the assembler was determined as described above. To validate these, we examined all reads mapped to the finished sequence of chromosome 21 (48) and determined how many incorrect mate pairs there were as a result of laboratory tracking errors and chimerism (two different segments of the genome cloned into the same plasmid), and how tight the distribution of insert sizes was for

those that were correct (Table 5). The standard deviations for all Celera libraries were quite small, less than 15% of the insert length, with the exception of a few 50-kbp libraries. The 2- and 10-kbp libraries contained less than 2% invalid mate pairs, whereas the 50-kbp libraries were somewhat higher (~10%). Thus, although the mate-pair information was not perfect, its accuracy was such that measuring valid, misoriented, and mis-separated pairs with respect to a given assembly was deemed to be a reliable instrument for validation purposes, especially when several mate pairs confirm or deny an ordering.

The clone coverage of the genome was 39 \times , meaning that any given base pair was, on average, contained in 39 clones or, equivalently, spanned by 39 mate-paired reads. Areas of low clone coverage or areas with a high proportion of invalid mate pairs would indicate potential assembly problems. We computed the coverage of each base in the assembly by valid mate pairs (Table 6). In summary, for scaffolds >30 kbp in length, less than 1% of the Celera assembly was in regions of less than 3 \times clone coverage. Thus, more than 99% of the assembly, including order and orientation, is strongly supported by this measure alone.

We examined the locations and number of all misoriented and mis-separated mates. In addition to doing this analysis on the CSA assembly (as of 1 October 2000), we also performed a study of the PFP assembly as of

5 September 2000 (30, 55b). In this latter case, Celera mate pairs had to be mapped to the PFP assembly. To avoid mapping errors due to high-fidelity repeats, the only pairs mapped were those for which both reads matched at only one location with less than 6% differences. A threshold was set such that sets of five or more simultaneously invalid mate pairs indicated a potential breakpoint, where the construction of the two assemblies differed. The graphic comparison of the CSA chromosome 21 assembly with the published sequence (Fig. 6A) serves as a validation of this methodology. Blue tick marks in the panels indicate breakpoints. There were a similar (small) number of breakpoints on both chromosome sequences. The exception was 12 sets of scaffolds in the Celera assembly (a total of 3% of the chromosome length in 212 single-contig scaffolds) that were mapped to the wrong positions because they were too small to be mapped reliably. Figures 6 and 7 and Table 6 illustrate the mate-pair differences and breakpoints between the two assemblies. There was a higher percentage of misoriented and mis-separated mate pairs in the large-insert libraries (50 kbp and BAC ends) than in the small-insert libraries in both assemblies (Table 6). The large-insert libraries are more likely to identify discrepancies simply because they span a larger segment of the genome. The graphic comparison between the two assemblies for chromosome 8 (Fig. 6, B and C) shows that there are many

Table 5. Mate-pair validation. Celera fragment sequences were mapped to the published sequence of chromosome 21. Each mate pair uniquely mapped was evaluated for correct orientation and placement (number

of mate pairs tested). If the two mates had incorrect relative orientation or placement, they were considered invalid (number of invalid mate pairs).

Library type	Library no.	Chromosome 21						Genome		
		Mean insert size (bp)	SD (bp)	SD/mean (%)	No. of mate pairs tested	No. of invalid mate pairs	% invalid	Mean insert size (bp)	SD (bp)	SD/mean (%)
2 kbp	1	2,081	106	5.1	3,642	38	1.0	2,082	90	4.3
	2	1,913	152	7.9	28,029	413	1.5	1,923	118	6.1
	3	2,166	175	8.1	4,405	57	1.3	2,162	158	7.3
10 kbp	4	11,385	851	7.5	4,319	80	1.9	11,370	696	6.1
	5	14,523	1,875	12.9	7,355	156	2.1	14,142	1,402	9.9
	6	9,635	1,035	10.7	5,573	109	2.0	9,606	934	9.7
	7	10,223	928	9.1	34,079	399	1.2	10,190	777	7.6
50 kbp	8	64,888	2,747	4.2	16	1	6.3	65,500	5,504	8.4
	9	53,410	5,834	10.9	914	170	18.6	53,311	5,546	10.4
	10	52,034	7,312	14.1	5,871	569	9.7	51,498	6,588	12.8
	11	52,282	7,454	14.3	2,629	213	8.1	52,282	7,454	14.3
	12	46,616	7,378	15.8	2,153	215	10.0	45,418	9,068	20.0
	13	55,788	10,099	18.1	2,244	249	11.1	53,062	10,893	20.5
	14	39,894	5,019	12.6	199	7	3.5	36,838	9,988	27.1
	15	48,931	9,813	20.1	144	10	6.9	47,845	4,774	10.0
BES	16	48,130	4,232	8.8	195	14	7.2	47,924	4,581	9.6
	17	106,027	27,778	26.2	330	16	4.8	152,000	26,600	17.5
	18	160,575	54,973	34.2	155	8	5.2	161,750	27,000	16.7
	19	164,155	19,453	11.9	642	44	6.9	176,500	19,500	11.05
Sum					102,894	2,768	2.7			
							(mean = 2.7)			

more breakpoints for the PFP assembly than for the Celera assembly. Figure 7 shows the breakpoint map (blue tick marks) for both assemblies of each chromosome in a side-by-side fashion. The order and orientation of Celera's assembly shows substantially fewer breakpoints except on the two finished chromosomes. Figure 7 also depicts large gaps (>10 kbp) in both assemblies as red tick marks. In the CSA assembly, the size of all gaps have been estimated on the basis of the mate-pair data. Breakpoints can be caused by structural polymorphisms, because the two assemblies were derived from different human genomes. They also reflect the unfinished nature of both genome assemblies.

3 Gene Prediction and Annotation

Summary. To enumerate the gene inventory, we developed an integrated, evidence-based approach named Otto. The evidence used to increase the likelihood of identifying genes includes regions conserved between the mouse and human genomes, similarity to ESTs or other mRNA-derived data, or similarity to other proteins. A comparison of Otto (combined Otto-RefSeq and Otto homology) with Genscan, a standard gene-prediction algorithm, showed greater sensitivity (0.78 versus 0.50) and specificity (0.93 versus 0.63) of Otto in the ability to define gene structure. Otto-predicted genes were complemented with a set of genes from three gene-prediction programs that exhibited weaker, but still significant, evidence that they may be expressed. Conservative criteria, requiring at least two lines of evidence, were used to define a set of 26,383 genes with good confidence that were used for more detailed analysis presented in the subsequent sections. Extensive manual curation to establish precise characterization of gene structure will be necessary to improve the results from this initial computational approach.

3.1 Automated gene annotation

A gene is a locus of cotranscribed exons. A single gene may give rise to multiple transcripts, and thus multiple distinct proteins with multiple functions, by means of alterna-

tive splicing and alternative transcription initiation and termination sites. Our cells are able to discern within the billions of base pairs of the genomic DNA the signals for initiating transcription and for splicing together exons separated by a few or hundreds of thousands of base pairs. The first step in characterizing the genome is to define the structure of each gene and each transcription unit.

The number of protein-coding genes in mammals has been controversial from the outset. Initial estimates based on reassociation data placed it between 30,000 to 40,000, whereas later estimates from the brain were >100,000 (56). More recent data from both the corporate and public sectors, based on extrapolations from EST, CpG island, and transcript density-based extrapolations, have not reduced this variance. The highest recent number of 142,634 genes emanates from a report from Incyte Pharmaceuticals, and is based on a combination of EST data and the association of ESTs with CpG islands (57). In stark contrast are three quite different, and much lower estimates: one of ~35,000 genes derived with genome-wide EST data and sampling procedures in conjunction with chromosome 22 data (58); another of 28,000 to 34,000 genes derived with a comparative methodology involving sequence conservation between humans and the puffer fish *Tetraodon nigroviridis* (59); and a figure of 35,000 genes, which was derived simply by extrapolating from the density of 770 known and predicted genes in the 67 Mbp of chromosomes 21 and 22, to the approximately 3-Gbp euchromatic genome.

The problem of computational identification of transcriptional units in genomic DNA sequence can be divided into two phases. The first is to partition the sequence into segments that are likely to correspond to individual genes. This is not trivial and is a weakness of most de novo gene-finding algorithms. It is also critical to determining the number of genes in the human gene inventory. The second challenge is to construct a gene model that reflects the probable structure of the transcript(s) encoded in the region. This can

be done with reasonable accuracy when a full-length cDNA has been sequenced or a highly homologous protein sequence is known. De novo gene prediction, although less accurate, is the only way to find genes that are not represented by homologous proteins or ESTs. The following section describes the methods we have developed to address these problems for the prediction of protein-coding genes.

We have developed a rule-based expert system, called Otto, to identify and characterize genes in the human genome (60). Otto attempts to simulate in software the process that a human annotator uses to identify a gene and refine its structure. In the process of annotating a region of the genome, a human curator examines the evidence provided by the computational pipeline (described below) and examines how various types of evidence relate to one another. A curator puts different levels of confidence in different types of evidence and looks for certain patterns of evidence to support gene annotation. For example, a curator may examine homology to a number of ESTs and evaluate whether or not they can be connected into a longer, virtual mRNA. The curator would also evaluate the strength of the similarity and the contiguity of the match, in essence asking whether any ESTs cross splice-junctions and whether the edges of putative exons have consensus splice sites. This kind of manual annotation process was used to annotate the *Drosophila* genome.

The Otto system can promote observed evidence to a gene annotation in one of two ways. First, if the evidence includes a high-quality match to the sequence of a known gene [here defined as a human gene represented in a curated subset of the RefSeq database (61)], then Otto can promote this to a gene annotation. In the second method, Otto evaluates a broad spectrum of evidence and determines if this evidence is adequate to support promotion to a gene annotation. These processes are described below.

Initially, gene boundaries are predicted on the basis of examination of sets of overlapping protein and EST matches generated by a computational pipeline (62). This pipeline searches the scaffold sequences against protein, EST, and genome-sequence databases to define regions of sequence similarity and runs three de novo gene-prediction programs.

To identify likely gene boundaries, regions of the genome were partitioned by Otto on the basis of sequence matches identified by BLAST. Each of the database sequences matched in the region under analysis was compared by an algorithm that takes into account both coordinates of the matching sequence, as well as the sequence type (e.g., protein, EST, and so forth). The results were used to group the matches into bins of related sequences that may define a gene and identify

Table 6. Genome-wide mate pair analysis of compartmentalized shotgun (CSA) and PFP assemblies.*

Genome library	CSA			PFP		
	% valid	% mis-oriented	% mis-separated†	% valid	% mis-oriented	% mis-separated†
2 kbp	98.5	0.6	1.0	95.7	2.0	2.3
10 kbp	96.7	1.0	2.3	81.9	9.6	8.6
50 kbp	93.9	4.5	1.5	64.2	22.3	13.5
BES	94.1	2.1	3.8	62.0	19.3	18.8
Mean	97.4	1.0	1.6	87.3	6.8	5.9

*Data for individual chromosomes can be found in Web fig. 3 on Science Online at www.sciencemag.org/cgi/content/full/291/5507/1304/DC1. †Mates are misseparated if their distance is >3 SD from the mean library size.

gene boundaries. During this process, multiple hits to the same region were collapsed to a coherent set of data by tracking the coverage of a region. For example, if a group of bases was represented by multiple overlapping ESTs, the union of these regions matched by the set of ESTs on the scaffold was marked as being supported by EST evidence. This resulted in a series of "gene bins," each of which was believed to contain a single gene. One weakness of this initial implementation of the algorithm was in predicting gene boundaries in regions of tandemly duplicated genes. Gene clusters frequently resulted in homologous neighboring genes

being joined together, resulting in an annotation that artificially concatenated these gene models.

Next, known genes (those with exact matches of a full-length cDNA sequence to the genome) were identified, and the region corresponding to the cDNA was annotated as a predicted transcript. A subset of the curated human gene set RefSeq from the National Center for Biotechnology Information (NCBI) was included as a data set searched in the computational pipeline. If a RefSeq transcript matched the genome assembly for at least 50% of its length at >92% identity, then the SIM4 (63) alignment of the RefSeq transcript to

the region of the genome under analysis was promoted to the status of an Otto annotation. Because the genome sequence has gaps and sequence errors such as frameshifts, it was not always possible to predict a transcript that agrees precisely with the experimentally determined cDNA sequence. A total of 6538 genes in our inventory were identified and transcripts predicted in this way.

Regions that have a substantial amount of sequence similarity, but do not match known genes, were analyzed by that part of the Otto system that uses the sequence similarity information to predict a transcript. Here, Otto

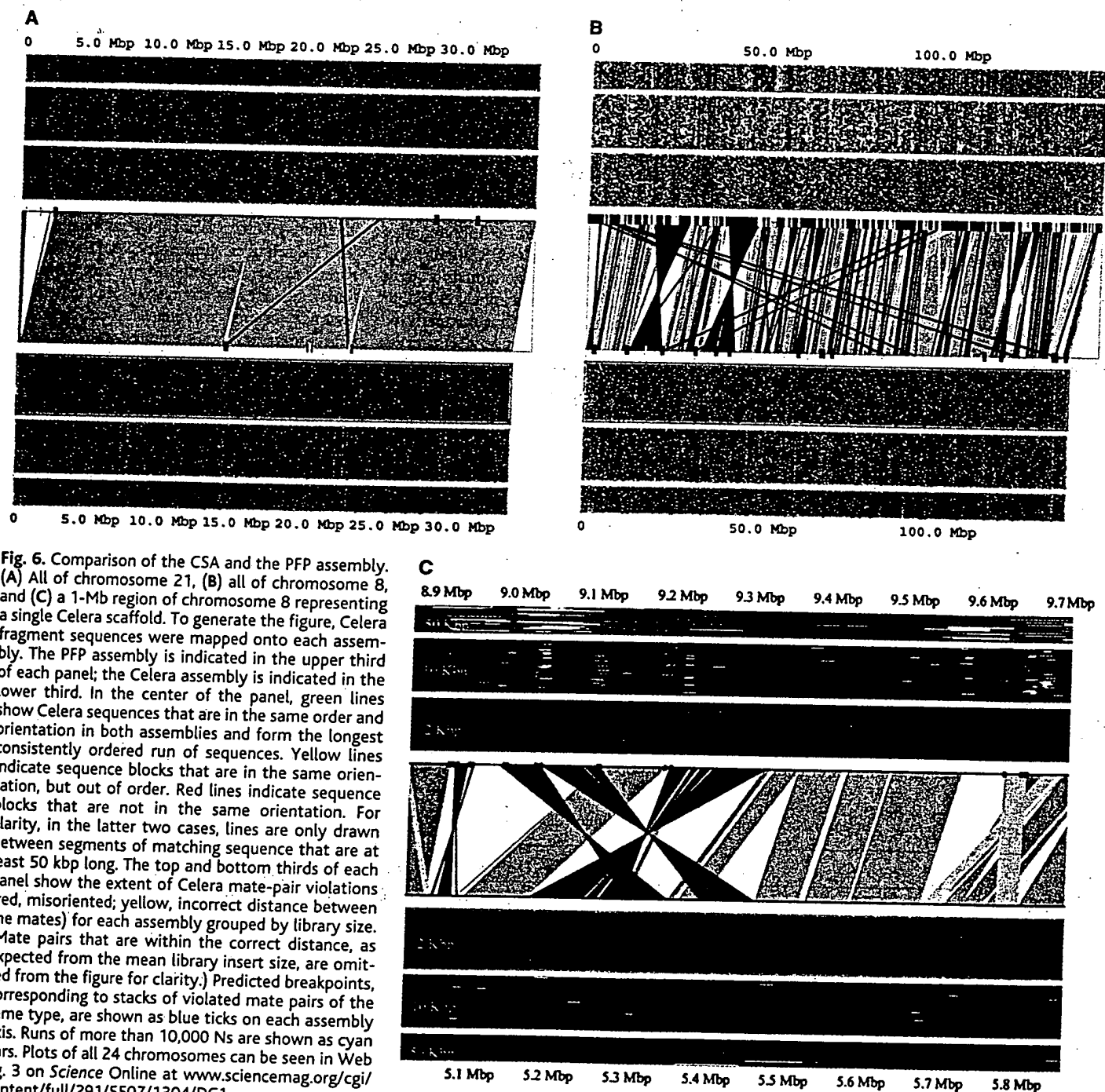


Fig. 6. Comparison of the CSA and the PFP assembly. (A) All of chromosome 21, (B) all of chromosome 8, and (C) a 1-Mb region of chromosome 8 representing a single Celera scaffold. To generate the figure, Celera fragment sequences were mapped onto each assembly. The PFP assembly is indicated in the upper third of each panel; the Celera assembly is indicated in the lower third. In the center of the panel, green lines show Celera sequences that are in the same order and orientation in both assemblies and form the longest consistently ordered run of sequences. Yellow lines indicate sequence blocks that are in the same orientation, but out of order. Red lines indicate sequence blocks that are not in the same orientation. For clarity, in the latter two cases, lines are only drawn between segments of matching sequence that are at least 50 kbp long. The top and bottom thirds of each panel show the extent of Celera mate-pair violations (red, misoriented; yellow, incorrect distance between the mates) for each assembly grouped by library size. (Mate pairs that are within the correct distance, as expected from the mean library insert size, are omitted from the figure for clarity.) Predicted breakpoints, corresponding to stacks of violated mate pairs of the same type, are shown as blue ticks on each assembly axis. Runs of more than 10,000 Ns are shown as cyan bars. Plots of all 24 chromosomes can be seen in Web fig. 3 on Science Online at www.sciencemag.org/cgi/content/full/291/5507/1304/DC1.

evaluates evidence generated by the computational pipeline, corresponding to conservation between mouse and human genomic DNA, similarity to human transcripts (ESTs

and cDNAs), similarity to rodent transcripts (ESTs and cDNAs), and similarity of the translation of human genomic DNA to known proteins to predict potential genes in the hu-

man genome. The sequence from the region of genomic DNA contained in a gene bin was extracted, and the subsequences supported by any homology evidence were marked (plus 100

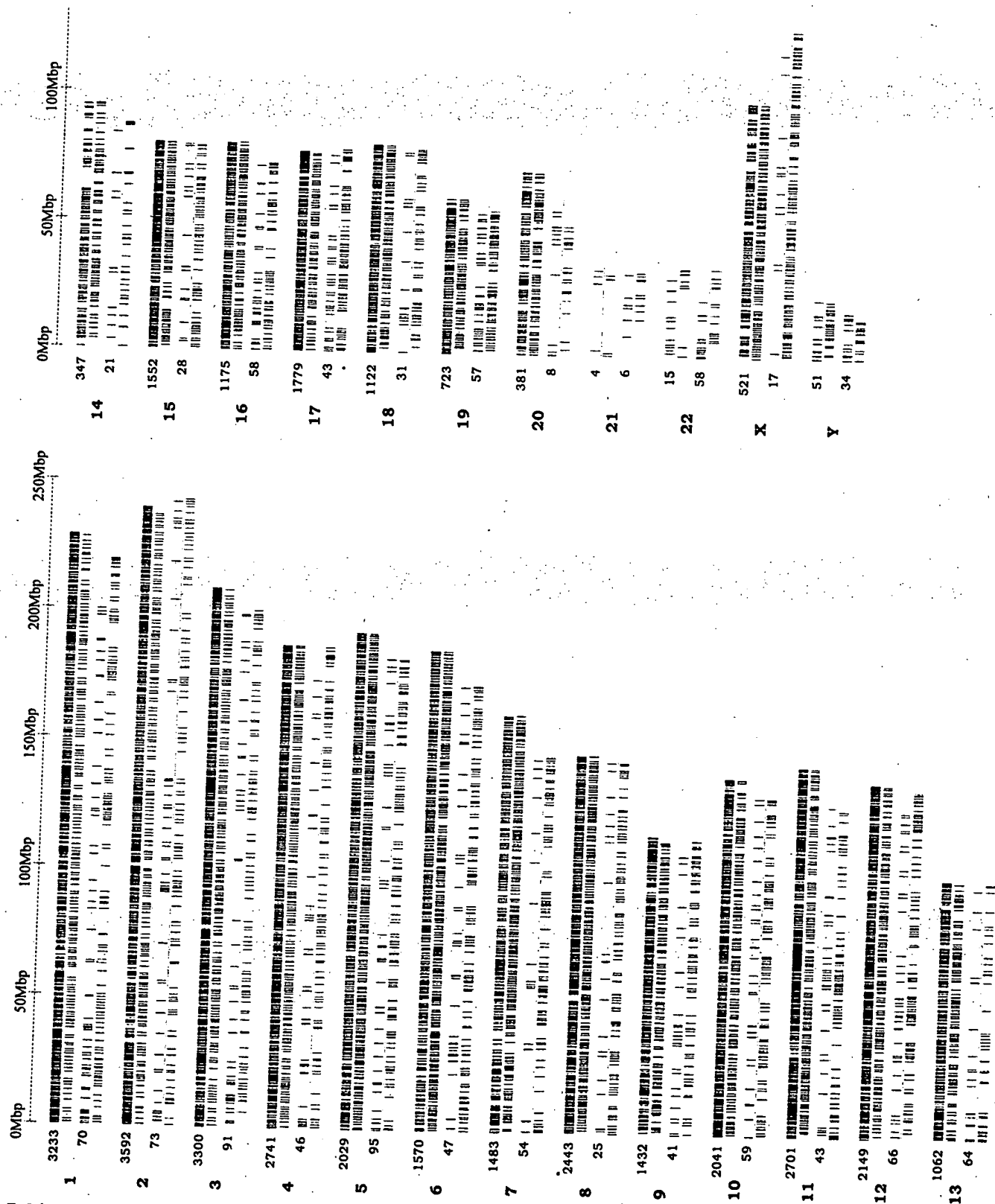


Fig. 7. Schematic view of the distribution of breakpoints and large gaps on all chromosomes. For each chromosome, the upper pair of lines represent the PFP assembly, and the lower pair of lines represent Celera's

assembly. Blue tick marks represent breakpoints, whereas red tick marks represent a gap of larger than 10,000 bp. The number of breakpoints per chromosome is indicated in black, and the chromosome numbers in red.

bases flanking these regions). The other bases in the region, those not covered by any homology evidence, were replaced by N's. This sequence segment, with high confidence regions represented by the consensus genomic sequence and the remainder represented by N's, was then evaluated by Genscan to see if a consistent gene model could be generated. This procedure simplified the gene-prediction task by first establishing the boundary for the gene (not a strength of most gene-finding algorithms), and by eliminating regions with no supporting evidence. If Genscan returned a plausible gene model, it was further evaluated before being promoted to an "Otto" annotation. The final Genscan predictions were often quite different from the prediction that Genscan returned on the same region of native genomic sequence. A weakness of using Genscan to refine the gene model is the loss of valid, small exons from the final annotation.

The next step in defining gene structures based on sequence similarity was to compare each predicted transcript with the homology-based evidence that was used in previous steps to evaluate the depth of evidence for each exon in the prediction. Internal exons were considered to be supported if they were covered by homology evidence to within ± 10 bases of their edges. For first and last exons, the internal edge was required to be within 10 bases, but the external edge was allowed greater latitude to allow for 5' and 3' untranslated regions (UTRs). To be retained, a prediction for a multi-exon gene must have evidence such that the total number of "hits," as defined above, divided by the number of exons in the prediction must be >0.66 or must correspond to a RefSeq sequence. A single-exon gene must be covered by at least three supporting hits (± 10 bases on each side), and these must cover the complete predicted open reading frame. For a single-exon gene, we also required that the Genscan prediction include both a start and a stop codon. Gene models that did not meet these criteria were disregarded, and

those that passed were promoted to Otto predictions. Homology-based Otto predictions do not contain 3' and 5' untranslated sequence. Although three de novo gene-finding programs [GRAIL, Genscan, and FgenesH (63)] were run as part of the computational analysis, the results of these programs were not directly used in making the Otto predictions. Otto predicted 11,226 additional genes by means of sequence similarity.

3.2 Otto validation

To validate the Otto homology-based process and the method that Otto uses to define the structures of known genes, we compared transcripts predicted by Otto with their corresponding (and presumably correct) transcript from a set of 4512 RefSeq transcripts for which there was a unique SIM4 alignment (Table 7). In order to evaluate the relative performance of Otto and Genscan, we made three comparisons. The first involved a determination of the accuracy of gene models predicted by Otto with only homology data other than the corresponding RefSeq sequence (Otto homology in Table 7). We measured the sensitivity (correctly predicted bases divided by the total length of the cDNA) and specificity (correctly predicted bases divided by the sum of the correctly and incorrectly predicted bases). Second, we examined the sensitivity and specificity of the Otto predictions that were made solely with the RefSeq sequence, which is the process that Otto uses to annotate known genes (Otto-RefSeq). And third, we determined the accuracy of the Genscan predictions corresponding to these RefSeq sequences. As expected, the alignment method (Otto-RefSeq) was the most accurate, and Otto-homology performed better than Genscan by both criteria. Thus, 6.1% of true RefSeq nucleotides were not represented in the Otto-refseq annotations and 2.7% of the nucleotides in the Otto-RefSeq transcripts were not contained in the original RefSeq transcripts. The discrepancies could come from legitimate differences between the Celera assembly and the RefSeq transcript due to polymorphisms, incomplete or incorrect data in the Celera assembly, errors introduced by Sim4 during the alignment process, or the presence of alternatively spliced forms in the data set used for the comparisons.

Because Otto uses an evidence-based approach to reconstruct genes, the absence of experimental evidence for intervening exons may inadvertently result in a set of exons that cannot be spliced together to give rise to a transcript. In such cases, Otto may "split genes" when in fact all the evidence should be combined into a single transcript. We also examined the tendency of these methods to incorrectly split gene predictions. These trends are shown in Fig. 8. Both RefSeq and homology-based predictions by Otto split known genes into fewer segments than Genscan alone.

3.3 Gene number

Recognizing that the Otto system is quite conservative, we used a different gene-prediction strategy in regions where the homology evidence was less strong. Here the results of de novo gene predictions were used. For these genes, we insisted that a predicted transcript have at least two of the following types of evidence to be included in the gene set for further analysis: protein, human EST, rodent EST, or mouse genome fragment matches. This final class of predicted genes is a subset of the predictions made by the three gene-finding programs that were used in the computational pipeline. For these, there was not sufficient sequence similarity information for Otto to attempt to predict a gene structure. The three de novo gene-finding programs resulted in about 155,695 predictions, of which ~76,410 were nonredundant (non-overlapping with one another). Of these, 57,935 did not overlap known genes or predictions made by Otto. Only 21,350 of the gene predictions that did not overlap Otto predictions were partially supported by at least one type of sequence similarity evidence, and 8619 were partially supported by two types of evidence (Table 8).

The sum of this number (21,350) and the number of Otto annotations (17,764), 39,114, is near the upper limit for the human gene complement. As seen in Table 8, if the requirement for other supporting evidence is made more stringent, this number drops rapidly so that demanding two types of evidence reduces the total gene number to 26,383 and demanding three types reduces it to ~23,000. Requiring that a prediction be supported by all four categories of evidence is too stringent because it would eliminate genes that encode novel proteins (members of currently undescribed protein families). No correction for pseudogenes has been made at this point in the analysis.

In a further attempt to identify genes that were not found by the autoannotation process or any of the de novo gene finders, we examined regions outside of gene predictions that were similar to the EST sequence, and where the EST matched the genomic sequence across a splice junction. After correcting for potential 3' UTRs of predicted genes, about 2500 such regions remained. Addition of a requirement for at least one of the following evidence types—homology to mouse genomic sequence fragments, rodent ESTs, or cDNAs—or similarity to a known protein reduced this number to 1010. Adding this to the numbers from the previous paragraph would give us estimates of about 40,000, 27,000, and 24,000 potential genes in the human genome, depending on the stringency of evidence considered. Table 8 illustrates the number of genes and presents the degree of

Table 7. Sensitivity and specificity of Otto and Genscan. Sensitivity and specificity were calculated by first aligning the prediction to the published RefSeq transcript, tallying the number (N) of uniquely aligned RefSeq bases. Sensitivity is the ratio of N to the length of the published RefSeq transcript. Specificity is the ratio of N to the length of the prediction. All differences are significant (Tukey HSD; $P < 0.001$).

Method	Sensitivity	Specificity
Otto (RefSeq only)*	0.939	0.973
Otto (homology)†	0.604	0.884
Genscan	0.501	0.633

*Refers to those annotations produced by Otto using only the Sim4-polished RefSeq alignment rather than an evidence-based Genscan prediction. †Refers to those annotations produced by supplying all available evidence to Genscan.

confidence based on the supporting evidence. Transcripts encoded by a set of 26,383 genes were assembled for further analysis. This set includes the 6538 genes predicted by Otto on the basis of matches to known genes, 11,226 transcripts predicted by Otto based on homology evidence, and 8619 from the subset of transcripts from de novo gene-prediction programs that have two types of supporting evidence. The 26,383 genes are illustrated along chromosome diagrams in Fig. 1. These are a very preliminary set of annotations and are subject to all the limitations of an automated process. Considerable refinement is still necessary to improve the accuracy of these transcript predictions. All the predictions and descriptions of genes and the associated evidence that we present are the product of completely computational processes, not expert curation. We have attempted to enumerate the genes in the human genome in such a way that we have different levels of confidence based on the amount of supporting evidence: known genes, genes with good protein or EST homology evidence, and de novo gene predictions confirmed by modest homology evidence.

3.4 Features of human gene transcripts

We estimate the average span for a "typical" gene in the human DNA sequence to be about 27,894 bases. This is based on the average span covered by RefSeq transcripts, used because it represents our highest confidence set.

The set of transcripts promoted to gene annotations varies in a number of ways. As can be seen from Table 8 and Fig. 9, transcripts predicted by Otto tend to be longer, having on average about 7.8 exons, whereas those promoted from gene-prediction programs average about 3.7 exons. The largest number of exons that we have identified in a transcript is 234 in the titin mRNA. Table 8 compares the amounts of evidence that sup-

port the Otto and other predicted transcripts. For example, one can see that a typical Otto transcript has 6.99 of its 7.81 exons supported by protein homology evidence. As would be expected, the Otto transcripts generally have more support than do transcripts predicted by the de novo methods.

4 Genome Structure

Summary. This section describes several of the noncoding attributes of the assembled genome sequence and their correlations with the predicted gene set. These include an analysis of G+C content and gene density in the context of cytogenetic maps of the genome, an enumerative analysis of CpG islands, and a brief description of the genome-wide repetitive elements.

4.1 Cytogenetic maps

Perhaps the most obvious, and certainly the most visible, element of the structure of the genome is the banding pattern produced by Giemsa stain. Chromosomal banding studies have revealed that about 17% to 20% of the human chromosome complement consists of C-bands, or constitutive heterochromatin (64). Much of this heterochromatin is highly polymorphic and consists of different families of alpha satellite DNAs with various higher order repeat structures (65). Many chromosomes have complex inter- and intrachromosomal duplications present in pericentromeric regions (66). About 5% of the sequence reads were identified as alpha satellite sequences; these were not included in the assembly.

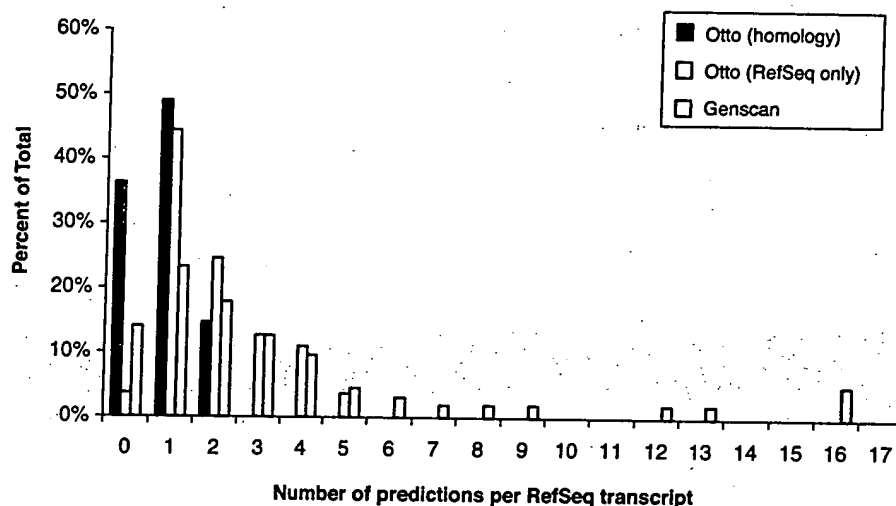


Fig. 8. Analysis of split genes resulting from different annotation methods. A set of 4512 Sim4-based alignments of RefSeq transcripts to the genomic assembly were chosen (see the text for criteria), and the numbers of overlapping Genscan, Otto (RefSeq only) annotations based solely on Sim4-polished RefSeq alignments, and Otto (homology) annotations (annotations produced by supplying all available evidence to Genscan) were tallied. These data show the degree to which multiple Genscan predictions and/or Otto annotations were associated with a single RefSeq transcript. The zero class for the Otto-homology predictions shown here indicates that the Otto-homology calls were made without recourse to the RefSeq transcript, and thus no Otto call was made because of insufficient evidence.

Table 8. Numbers of exons and transcripts supported by various types of evidence for Otto and de novo gene prediction methods. Highlighted cells indicate the gene sets analyzed in this paper (boldface, set of genes selected for protein analysis; italic, total set of accepted de novo predictions).

		Total	Types of evidence				No. of lines of evidence*			
			Mouse	Rodent	Protein	Human	≥1	≥2	≥3	≥4
Otto	Number of transcripts	17,969	17,065	14,881	15,477	16,374	17,968†	17,501	15,877	12,451
	Number of exons	141,218	111,174	89,569	108,431	118,869	140,710	127,955	99,574	59,804
De novo	Number of transcripts	58,032	14,463	5,094	8,043	9,220	21,350	8,619	4,947	1,904
	Number of exons	319,935	48,594	19,344	26,264	40,104	79,148	31,130	17,508	6,520
No. of exons per transcript	Otto	7.84	5.77	6.01	6.99	7.24	7.81	7.19	6.00	4.28
	De novo	5.53	3.17	3.80	3.27	4.36	3.7	3.56	3.42	3.16

*Four kinds of evidence (conservation in 3× mouse genomic DNA, similarity to human EST or cDNA, similarity to rodent EST or cDNA, and similarity to known proteins) were considered to support gene predictions from the different methods. The use of evidence is quite liberal, requiring only a partial match to a single exon of predicted transcript. †This number includes alternative splice forms of the 17,764 genes mentioned elsewhere in the text.

Examination of pericentromeric regions is ongoing.

The remaining ~80% of the genome, the euchromatic component, is divisible into G-, R-, and T-bands (67). These cytogenetic bands have been presumed to differ in their nucleotide composition and gene density, although we have been unable to determine precise band boundaries at the molecular level. T-bands are the most G+C- and gene-rich, and G-bands are G+C-poor (68). Bernardi has also offered a description of the euchromatin at the molecular level as long stretches of DNA of differing base composition, termed isochores (denoted L, H1, H2, and H3), which are >300 kbp in length (69). Bernardi defined the L (light) isochores as G+C-poor (<43%), whereas the H (heavy) isochores fall into three G+C-rich classes representing 24, 8, and 5% of the genome. Gene concentration has been claimed to be very low in the L isochores and 20-fold more enriched in the H2 and H3 isochores (70). By examining contiguous 50-kbp windows of G+C content across the assembly, we found that regions of G+C content >48% (H3 isochores) averaged 273.9 kbp in length, those with G+C content between 43 and 48% (H1+H2 isochores) averaged 202.8 kbp in length, and the average span of regions with <43% (L isochores) was 1078.6 kbp. The correlation between G+C content and gene density was also examined in 50-kbp windows along the assembled sequence (Table 9 and Figs. 10 and 11). We found that the density of genes was greater in regions of high G+C than in regions of low G+C content, as expected. However, the correlation between G+C content and gene density was not as skewed as previously predicted (69). A higher proportion of genes were located in the G+C-poor regions than had been expected.

Chromosomes 17, 19, and 22, which have a disproportionate number of H3-containing bands, had the highest gene density (Table 10). Conversely, of the chromosomes that we

found to have the lowest gene density, X, 4, 18, 13, and Y, also have the fewest H3 bands. Chromosome 15, which also has few H3 bands, did not have a particularly low gene density in our analysis. In addition, chromosome 8, which we found to have a low gene density, does not appear to be unusual in its H3 banding.

How valid is Ohno's postulate (71) that mammalian genomes consist of oases of genes in otherwise essentially empty deserts? It appears that the human genome does indeed contain deserts, or large, gene-poor regions. If we define a desert as a region >500 kbp without a gene, then we see that 605 Mbp, or about 20% of the genome, is in deserts. These are not uniformly distributed over the various chromosomes. Gene-rich chromosomes 17, 19, and 22 have only about 12% of their collective 171 Mbp in deserts, whereas gene-poor chromosomes 4, 13, 18, and X have 27.5% of their 492 Mbp in deserts (Table 11). The apparent lack of predicted genes in these regions does not necessarily imply that they are devoid of biological function.

4.2 Linkage map

Linkage maps provide the basis for genetic analysis and are widely used in the study of the inheritance of traits and in the positional cloning of genes. The distance metric, centimorgans (cM), is based on the recombination rate between homologous chromosomes during meio-

sis. In general, the rate of recombination in females is greater than that in males, and this degree of map expansion is not uniform across the genome (72). One of the opportunities enabled by a nearly complete genome sequence is to produce the ultimate physical map, and to fully analyze its correspondence with two other maps that have been widely used in genome and genetic analysis: the linkage map and the cytogenetic map. This would close the loop between the mapping and sequencing phases of the genome project.

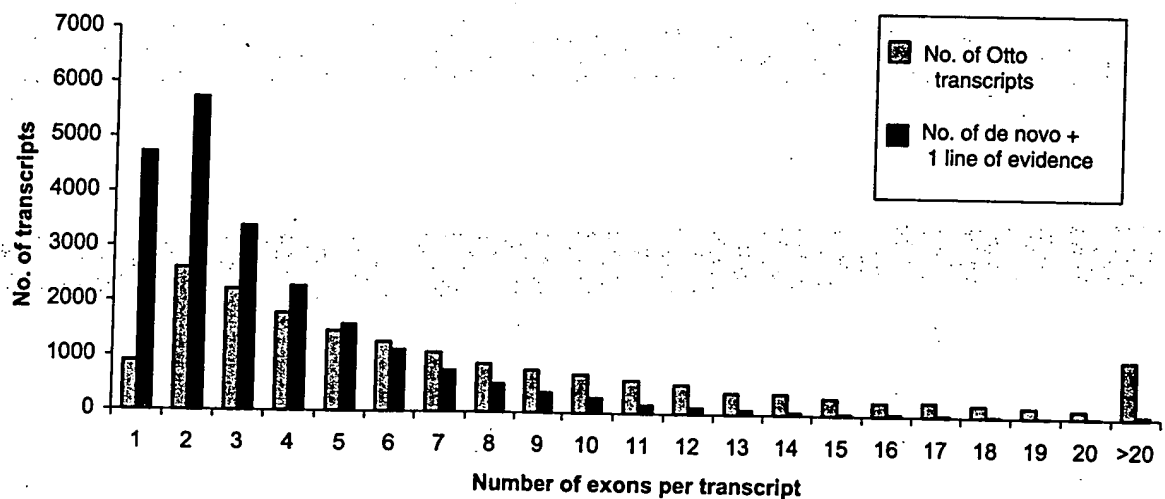
We mapped the location of the markers that constitute the Genethon linkage map to the genome. The rate of recombination, expressed as cM per Mbp, was calculated for 3-Mbp windows as shown in Table 12. Higher rates of recombination in the telomeric region of the chromosomes have been previously documented (73). From this mapping result, there is a difference of 4.99 between lowest rates and highest rates and the largest difference of 4.4 between males and females (4.99 to 0.47 on chromosome 16). This indicates that the variability in recombination rates among regions of the genome exceeds the differences in recombination rates between males and females. The human genome has recombination hotspots, where recombination rates vary fivefold or more over a space of 1 kbp, so the picture one gets of the magnitude of variability in recombination rate will depend on the size of the window

Table 9. Characteristics of G+C in isochores.

Isochore	G+C (%)	Fraction of genome		Fraction of genes	
		Predicted*	Observed	Predicted*	Observed
H3	>48	5	9.5	37	24.8
H1/H2	43-48	25	21.2	32	26.6
L	<43	67	69.2	31	48.5

*The predictions were based on Bernardi's definitions (70) of the isochore structure of the human genome.

Fig. 9. Comparison of the number of exons per transcript between the 17,968 Otto transcripts and 21,350 de novo transcript predictions with at least one line of evidence that do not overlap with an Otto prediction. Both sets have the highest number of transcripts in the two-exon category, but the de novo gene predictions are skewed much more toward smaller transcripts. In the Otto set, 19.7% of the transcripts have one or two exons, and 5.7% have more than 20. In the de novo set, 49.3% of the transcripts have one or two exons, and 0.2% have more than 20.



examined. Unfortunately, too few meiotic crossovers have occurred in Centre d'Étude du Polymorphisme Humain (CEPH) and other reference families to provide a resolution any finer than about 3 Mbp. The next challenge will be to determine a sequence basis of recombination at the chromosomal level. An accurate predictor for the rate for variation in recombination rates between any pair of markers would be extremely useful in designing markers to narrow a region of linkage, such as in positional cloning projects.

4.3 Correlation between CpG islands and genes

CpG islands are stretches of unmethylated DNA with a higher frequency of CpG dinucleotides when compared with the entire genome (74). CpG islands are believed to preferentially occur at the transcriptional start of genes, and it has been observed that most housekeeping genes have CpG islands at the 5' end of the transcript (75, 76). In addition, experimental evidence indicates that CpG island methylation is correlated with gene inactivation (77) and has been shown to be important during gene imprinting (78) and tissue-specific gene expression (79).

Experimental methods have been used that resulted in an estimate of 30,000 to 45,000 CpG islands in the human genome (74, 80) and an estimate of 499 CpG islands on human chromosome 22 (81). Larsen *et al.* (76) and Gardiner-Garden and Frommer (75) used a computational method to identify CpG islands and defined them as regions of DNA of >200 bp that have a G+C content of >50% and a ratio of observed

versus expected frequency of CG dinucleotide ≥ 0.6 .

It is difficult to make a direct comparison of experimental definitions of CpG islands with computational definitions because computational methods do not consider the methylation state of cytosine and experimental methods do not directly select regions of high G+C content. However, we can determine the correlation of CpG island with gene starts, given a set of annotated genomic transcripts and the whole genome sequence. We have analyzed the publicly available annotation of chromosome 22, as well as using the entire human genome in our assembly and the computationally annotated genes. A variation of the CpG island computation was compared with Larsen *et al.* (76). The main differences are that we use a sliding window of 200 bp, consecutive windows are merged only if they overlap, and we recompute the CpG value upon merging, thus rejecting any potential island if it scores less than the threshold.

To compute various CpG statistics, we used two different thresholds of CG dinucleotide likelihood ratio. Besides using the original threshold of 0.6 (method 1), we used a higher threshold of CG dinucleotide likelihood ratio of 0.8 (method 2), which results in the number of CpG islands on chromosome 22 close to the number of annotated genes on this chromosome. The main results are summarized in Table 13. CpG islands computed with method 1 predicted only 2.6% of the CSA sequence as CpG, but 40% of the gene starts (start codons) are contained inside a

CpG island. This is comparable to ratios reported by others (82). The last two rows of the table show the observed and expected average distance, respectively, of the closest CpG island from the first exon. The observed average closest CpG islands are smaller than the corresponding expected distances, confirming an association between CpG island and the first exon.

We also looked at the distribution of CpG island nucleotides among various sequence classes such as intergenic regions, introns, exons, and first exons. We computed the likelihood score for each sequence class as the ratio of the observed fraction of CpG island nucleotides in that sequence class and the expected fraction of CpG island nucleotides in that sequence class. The result of applying method 1 on CSA were scores of 0.89 for intergenic region, 1.2 for intron, 5.86 for exon, and 13.2 for first exon. The same trend was also found for chromosome 22 and after the application of a higher threshold (method 2) on both data sets. In sum, genome-wide analysis has extended earlier analysis and suggests a strong correlation between CpG islands and first coding exons.

4.4 Genome-wide repetitive elements

The proportion of the genome covered by various classes of repetitive DNA is presented in Table 14. We observed about 35% of the genome in these repeat classes, very similar to values reported previously (83). Repetitive sequence may be underrepresented in the Celera assembly as a result of incomplete repeat resolution, as discussed above. About 8% of the scaffold length is in gaps, and we expect that much of this is repetitive sequence. Chromosome 19 has the highest repeat density (57%), as well as the highest gene density (Table 10). Of interest, among the different classes of repeat elements, we observe a clear association of Alu elements and gene density, which was not observed between LINES and gene density.

5 Genome Evolution

Summary. The dynamic nature of genome evolution can be captured at several levels. These include gene duplications mediated by RNA intermediates (retrotransposition) and segmental genomic duplications. In this section, we document the genome-wide occurrence of retrotransposition events generating functional (intronless paralogs) or inactive genes (pseudogenes). Genes involved in translational processes and nuclear regulation account for nearly 50% of all intronless paralogs and processed pseudogenes detected in our survey. We have also cataloged the extent of segmental genomic duplication and provide evidence for 1077 duplicated blocks covering 3522 distinct genes.

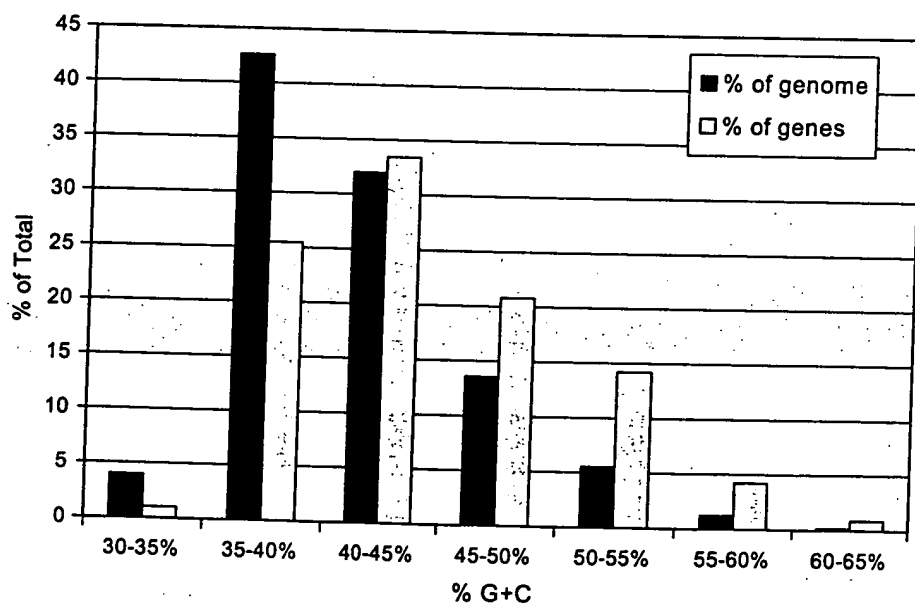


Fig. 10. Relation between G+C content and gene density. The blue bars show the percent of the genome (in 50-kbp windows) with the indicated G+C content. The percent of the total number of genes associated with each G+C bin is represented by the yellow bars. The graph shows that about 5% of the genome has a G+C content of between 50 and 55%, but that this portion contains nearly 15% of the genes.

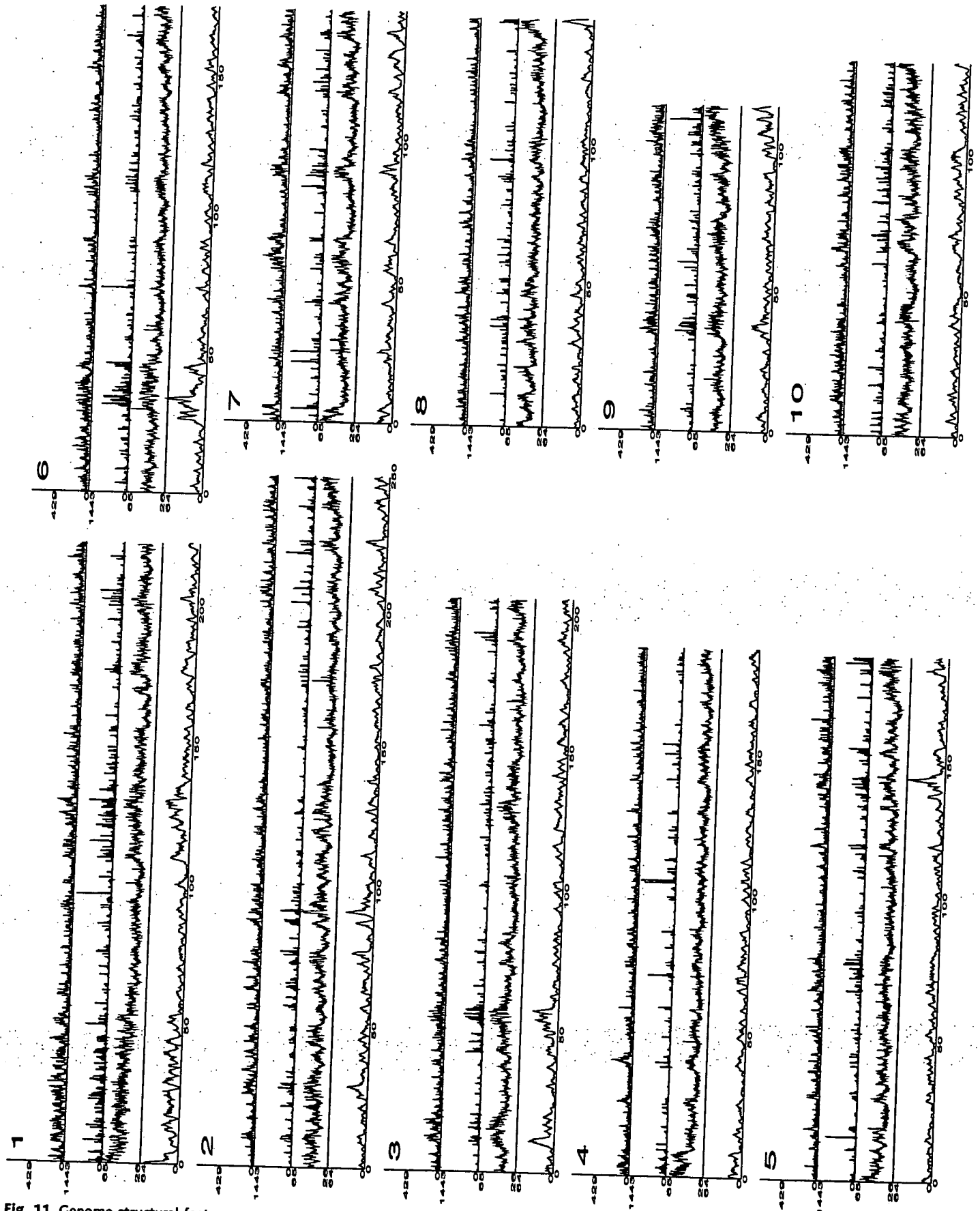


Fig. 11. Genome structural features.

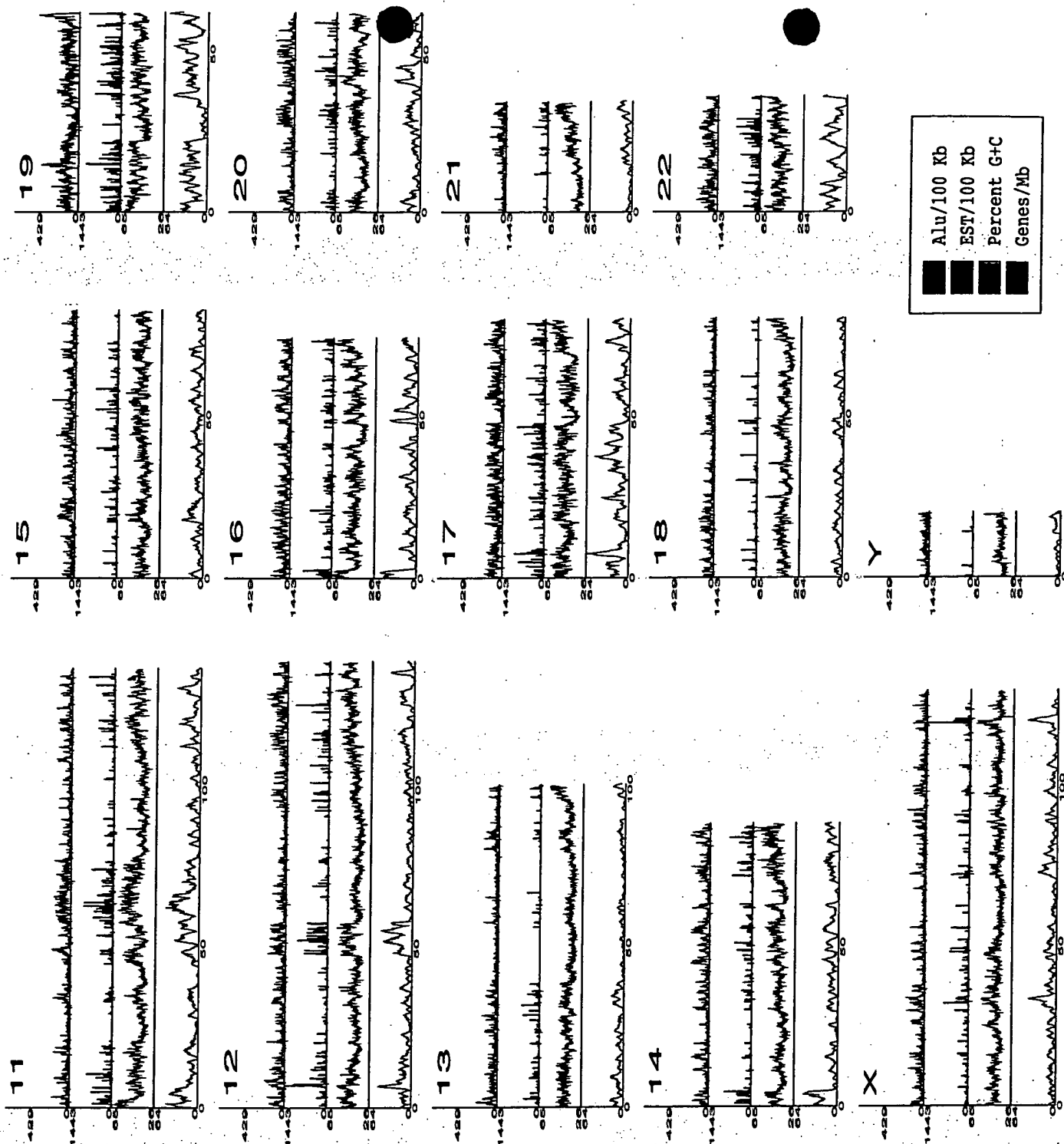


Fig. 11 (continued). Relation among gene density (orange), G+C content (green), EST density (blue), and Alu density (pink) along the lengths of each of the chromosomes. Gene density was calculated in 1-Mbp win-

dows. The percent of G+C nucleotides was calculated in 100-kbp windows. The number of ESTs and Alu elements is shown per 100-kbp window.

5.1 Retrotransposition in the human genome

Retrotransposition of processed mRNA transcripts into the genome results in functional genes, called intronless paralogs, or inactivated genes (pseudogenes). A paralog refers to a gene that appears in more than one copy in a given organism as a result of

a duplication event. The existence of both intron-containing and intronless forms of genes encoding functionally similar or identical proteins has been previously described (84, 85). Cataloging these evolutionary events on the genomic landscape is of value in understanding the functional consequences of such gene-duplication

events in cellular biology. Identification of conserved intronless paralogs in the mouse or other mammalian genomes should provide the basis for capturing the evolutionary chronology of these transposition events and provide insights into gene loss and accretion in the mammalian radiation.

A set of proteins corresponding to all 901

Table 10. Features of the chromosomes. De novo/any refers to the union of de novo predictions that do not overlap Otto predictions and have at least one other type of supporting evidence; de novo/2x refers to the union of de novo predictions that do not overlap Otto predictions and have at least two types of evidence. Deserts are regions of sequence with no annotated genes.

Chr.	Base composition					Gene prediction*					Gene density (genes/Mbp)										
	Size (Mbp)	No. of scaffolds	Largest scaffold (Mbp)	No. of scaffolds >500 kbp	Se-quence covered by scaffolds >500 kbp	% of total se-quence in scaffolds >500 kbp	% repeat	% GC	No of CpG islands	Otto	De novo/any	De novo/2X	Total (Otto + de novo/any)	Total (Otto + de novo/any)	Se-quence in deserts >500/ kbp	Se-quence in deserts >1 Mbp	Otto	De novo/any	De novo/2X	Otto + de novo/any	Otto + de novo/2X
1	220	2,549	11	82	192	88	37	42	2,335	1,743	1,710	710	3,453	2,453	29	6	8	8	3	16	11
2	240	3,263	13	78	217	91	36	40	1,703	1,183	1,771	633	2,954	1,816	55	19	5	7	2	12	7
3	200	3,532	7	78	173	87	37	40	1,271	1,013	1,414	598	2,427	1,611	50	12	5	7	3	12	8
4	186	2,180	10	70	169	91	37	38	1,081	696	1,165	449	1,861	1,145	55	18	4	6	2	10	6
5	182	3,231	11	63	163	89	37	40	1,302	892	1,244	474	2,136	1,366	46	15	5	7	2	11	7
6	172	1,713	13	58	160	93	37	40	1,384	943	1,314	524	2,257	1,467	38	9	6	7	3	13	8
7	146	1,326	14	53	130	89	38	40	1,406	759	1,072	460	1,831	1,219	26	12	5	7	3	12	8
8	146	1,772	11	54	135	92	36	40	948	583	977	357	1,560	940	33	6	4	7	2	11	6
9	113	1,616	8	40	101	89	38	41	1,315	689	848	329	1,537	1,018	22	9	6	7	3	13	8
10	130	2,005	9	55	116	89	36	42	1,087	685	968	342	1,653	1,027	21	8	5	7	2	12	7
11	132	2,814	9	44	116	88	39	42	1,461	1,051	1,134	535	2,185	1,586	27	9	8	8	4	16	12
12	134	2,614	8	51	117	87	38	41	1,131	925	936	417	1,861	1,342	24	9	7	7	3	14	10
13	99	1,038	13	34	91	91	36	38	644	341	691	241	1,032	582	31	16	4	7	2	10	5
14	87	576	11	16	83	95	40	41	913	583	700	290	1,283	873	34	20	7	8	3	14	10
15	80	1,747	8	31	70	87	37	42	722	558	640	246	1,198	804	8	1	7	8	3	15	10
16	75	1,520	8	27	62	82	40	44	1,533	748	673	247	1,421	995	13	3	10	9	3	19	12
17	78	1,683	6	40	61	78	39	45	1,489	897	648	313	1,545	1,210	15	6	12	8	4	19	15
18	79	1,333	13	18	72	92	36	40	510	283	543	189	826	472	21	10	4	7	2	10	6
19	58	2,282	3	31	38	67	57	49	2,804	1,141	534	268	1,675	1,409	3	0	20	9	4	29	23
20	61	580	14	17	58	94	41	44	997	517	469	180	986	697	7	1	8	7	3	16	11
21	33	358	10	6	32	96	38	41	519	184	265	102	449	286	15	9	6	8	3	13	8
22	36	333	11	12	32	88	44	48	1,173	494	341	147	835	641	3	0	14	9	4	23	17
X	128	1,346	4	91	93	73	46	39	726	605	860	387	1,465	992	29	8	5	6	3	11	7
Y	19	638	2	10	12	65	50	39	65	55	155	49	210	104	4	2	3	8	2	11	5
total	2907	53,591	9	1,059	2,490	87	40	41	28,519	17,764	21,350	8,619	39,114	26,383	606	208	7	7	3	14	9
avg.	116	2,144	9	44	104	87	40	41	1,160	714	812	333	1,526	1,047	25	9	7	7	3	14	9

Chromosomal assignment unknown.

*Chromosomal assignment unknown.

Pseudogenes

A pseudogene is a nonfunctional copy that is very similar to a normal gene but that has been altered slightly so that it is not ex-

pressed. We developed a method for the preliminary analysis of processed pseudogenes in the human genome as a starting point in elucidating the ongoing evolutionary forces

Otto-predicted, single-exon genes were subjected to BLAST analysis against the proteins encoded by the remaining multiexon predicted transcripts. Using homology criteria of 70% sequence identity over 90% of the length, we identified 298 instances of single-to multi-exon correspondence. Of these 298 sequences, 97 were represented in the GenBank data set of experimentally validated full-length genes at the stringency specified and were verified by manual inspection.

We believe that these 97 cases may represent intronless paralogs (see Web table 1 on *Science* Online at www.sciencemag.org/cgi/content/full/291/5507/1304/DC1) of known genes. Most of these are flanked by direct repeat sequences, although the precise nature of these repeats remains to be determined. All of the cases for which we have high confidence contain polyadenylated [poly(A)] tails characteristic of retrotransposition.

Recent publications describing the phenomenon of functional intronless paralogs speculate that retrotransposition may serve as a mechanism used to escape X-chromosomal inactivation (84, 86). We do not find a bias toward X chromosome origination of these retrotransposed genes; rather, the results show a random chromosome distribution of both the intron-containing and corresponding intronless paralogs. We also have found several cases of retrotransposition from a single source chromosome to multiple target chromosomes. Interesting examples include the retrotransposition of a five exon-containing ribosomal protein L21 gene on chromosome 13 onto chromosomes 1, 3, 4, 7, 10, and 14, respectively. The size of the source genes can also show variability. The largest example is the 31-exon diacylglycerol kinase zeta gene on chromosome 11 that has an intronless paralog on chromosome 13. Regardless of route, retrotransposition with subsequent gene changes in coding or noncoding regions that lead to different functions or expression patterns, represents a key route to providing an enhanced functional repertoire in mammals (87).

Our preliminary set of retrotransposed intronless paralogs contains a clear overrepresentation of genes involved in translational processes (40% ribosomal proteins and 10% translation elongation factors) and nuclear regulation (HMG nonhistone proteins, 4%), as well as metabolic and regulatory enzymes. EST matches specific to a subset of intronless paralogs suggest expression of these intronless paralogs. Differences in the upstream regulatory sequences between the source genes and their intronless paralogs could account for differences in tissue-specific gene expression. Defining which, if any, of these processed genes are functionally expressed and translated will require further elucidation and experimental validation.

Table 11. Genome overview.

Size of the genome (including gaps)	2.91 Gbp
Size of the genome (excluding gaps)	2.66 Gbp
Longest contig	1.99 Mbp
Longest scaffold	14.4 Mbp
Percent of A+T in the genome	54
Percent of G+C in the genome	38
Percent of undetermined bases in the genome	9
Most GC-rich 50 kb	Chr. 2 (66%)
Least GC-rich 50 kb	Chr. X (25%)
Percent of genome classified as repeats	35
Number of annotated genes	26,383
Percent of annotated genes with unknown function	42
Number of genes (hypothetical and annotated)	39,114
Percent of hypothetical and annotated genes with unknown function	59
Gene with the most exons	Titin (234 exons)
Average gene size	27 kbp
Most gene-rich chromosome	Chr. 19 (23 genes/Mb)
Least gene-rich chromosomes	Chr. 13 (5 genes/Mb), Chr. Y (5 genes/Mb)
Total size of gene deserts (>500 kb with no annotated genes)	605 Mbp
Percent of base pairs spanned by genes	25.5 to 37.8*
Percent of base pairs spanned by exons	1.1 to 1.4*
Percent of base pairs spanned by introns	24.4 to 36.4*
Percent of base pairs in intergenic DNA	74.5 to 63.6*
Chromosome with highest proportion of DNA in annotated exons	Chr. 19 (9.33)
Chromosome with lowest proportion of DNA in annotated exons	Chr. Y (0.36)
Longest intergenic region (between annotated + hypothetical genes)	Chr. 13 (3,038,416 bp)
Rate of SNP variation	1/1250 bp

*In these ranges, the percentages correspond to the annotated gene set (26,383 genes) and the hypothetical + annotated gene set (39,114 genes), respectively.

Table 12. Rate of recombination per physical distance (cM/Mb) across the genome. Genethon markers were placed on CSA-mapped assemblies, and then relative physical distances and rates were calculated in 3-Mb windows for each chromosome. NA, not applicable.

Chrom.	Male			Sex-average			Female		
	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.
1	2.60	1.12	0.23	2.81	1.42	0.52	3.39	1.76	0.68
2	2.23	0.78	0.33	2.65	1.12	0.54	3.17	1.40	0.61
3	2.55	0.86	0.23	2.40	1.07	0.42	2.71	1.30	0.33
4	1.66	0.67	0.15	2.06	1.04	0.60	2.50	1.40	0.77
5	2.00	0.67	0.18	1.87	1.08	0.42	2.26	1.43	0.62
6	1.97	0.71	0.28	2.57	1.12	0.37	3.47	1.67	0.64
7	2.34	1.16	0.48	1.67	1.17	0.47	2.27	1.21	0.34
8	1.83	0.73	0.14	2.40	1.05	0.46	3.44	1.36	0.43
9	2.01	0.99	0.53	1.95	1.32	0.77	2.63	1.66	0.82
10	3.73	1.03	0.22	3.05	1.29	0.66	2.84	1.51	0.76
11	1.43	0.72	0.31	2.13	0.99	0.47	3.10	1.32	0.49
12	4.12	0.76	0.26	3.35	1.16	0.49	2.93	1.55	0.59
13	1.60	0.75	0.01	1.87	0.95	0.17	2.49	1.19	0.32
14	3.15	0.98	0.18	2.65	1.30	0.62	3.14	1.63	0.75
15	2.28	0.94	0.34	2.31	1.22	0.42	2.53	1.56	0.54
16	1.83	1.00	0.47	2.70	1.55	0.63	4.99	2.32	1.12
17	3.87	0.87	0.00	3.54	1.35	0.54	4.19	1.83	0.94
18	3.12	1.37	0.86	3.75	1.66	0.43	4.35	2.24	0.72
19	3.02	0.97	0.10	2.57	1.41	0.49	2.89	1.75	0.87
20	3.64	0.89	0.00	2.79	1.50	0.83	3.31	2.15	1.34
21	3.23	1.26	0.69	2.37	1.62	1.08	2.58	1.90	1.18
22	1.25	1.10	0.84	1.88	1.41	1.08	3.73	2.08	0.93
X	NA	NA	NA	NA	NA	NA	3.12	1.64	0.72
Y	NA	NA	NA	NA	NA	NA	NA	NA	NA
Genome	4.12	0.88	0.00	3.75	1.22	0.17	4.99	1.55	0.32

that account for gene inactivation. The general structural characteristics of these processed pseudogenes include the complete lack of intervening sequences found in the functional counterparts, a poly(A) tract at the 3' end, and direct repeats flanking the pseudogene sequence. Processed pseudogenes occur as a result of retrotransposition, whereas unprocessed pseudogenes arise from segmental genome duplication.

We searched the complete set of Otto-predicted transcripts against the genomic sequence by means of BLAST. Genomic regions corresponding to all Otto-predicted transcripts were excluded from this analysis. We identified 2909 regions matching with greater than 70% identity over at least 70% of the length of the transcripts that likely represent processed pseudogenes. This number is probably an underestimate because specific methods to search for pseudogenes were not used.

We looked for correlations between structural elements and the propensity for retrotransposition in the human genome. GC content and transcript length were compared between the genes with processed

pseudogenes (1177 source genes) versus the remainder of the predicted gene set. Transcripts that give rise to processed pseudogenes have shorter average transcript length (1027 bp versus 1594 bp for the Otto set) as compared with genes for which no pseudogene was detected. The overall GC content did not show any significant difference, contrary to a recent report (88). There is a clear trend in gene families that are present as processed pseudogenes. These include ribosomal proteins (67%), lamin receptors (10%), translation elongation factor alpha (5%), and HMG-non-histone proteins (2%). The increased occurrence of retrotransposition (both intronless paralogs and processed pseudogenes) among genes involved in translation and nuclear regulation may reflect an increased transcriptional activity of these genes.

5.3 Gene duplication in the human genome

Building on a previously published procedure (27), we developed a graph-theoretic algorithm, called Lek, for grouping the predicted human protein set into protein families (89).

The complete clusters that result from the Lek clustering provide one basis for comparing the role of whole-genome or chromosomal duplication in protein family expansion as opposed to other means, such as tandem duplication. Because each complete cluster represents a closed and certain island of homology, and because Lek is capable of simultaneously clustering protein complements of several organisms, the number of proteins contributed by each organism to a complete cluster can be predicted with confidence depending on the quality of the annotation of each genome. The variance of each organism's contribution to each cluster can then be calculated, allowing an assessment of the relative importance of large-scale duplication versus smaller-scale, organism-specific expansion and contraction of protein families, presumably as a result of natural selection operating on individual protein families within an organism. As can be seen in Fig. 12, the large variance in the relative numbers of human as compared with *D. melanogaster* and *Caenorhabditis elegans* proteins in complete clusters may be explained by multiple events of relative expansions in gene families in each of the three animal genomes. Such expansions would give rise to the distribution that shows a peak at 1:1 in the ratio for human-worm or human-fly clusters with the slope spread covering both human and fly/worm predominance, as we observed (Fig. 12). Furthermore, there are nearly as many clusters where worm and fly proteins predominate despite the larger numbers of proteins in the human. At face value, this analysis suggests that natural selection acting on individual protein families has been a major force driving the expansion of at least some elements of the human protein set. However, in our analysis, the difference between an ancient whole-genome duplication followed by loss, versus piecemeal duplication, cannot be easily distinguished. In order to differentiate these scenarios, more extended analyses were performed.

5.4 Large-scale duplications

Using two independent methods, we searched for large-scale duplications in the human genome. First, we describe a protein family-based method that identified highly conserved blocks of duplication. We then describe our comprehensive method for identifying all interchromosomal block duplications. The latter method identified a large number of duplicated chromosomal segments covering parts of all 24 chromosomes.

The first of the methods is based on the idea of searching for blocks of highly conserved homologous proteins that occur in more than one location on the genome. For this comparison, two genes were considered equivalent if their protein products were de-

Table 13. Characteristics of CpG islands identified in chromosome 22 (34-Mbp sequence length) and the whole genome (2.9-Gbp sequence length) by means of two different methods. Method 1 uses a CG likelihood ratio of ≥ 0.6 . Method 2 uses a CG likelihood ratio of ≥ 0.8 .

	Chromosome 22		Whole genome (CS assembly)	
	Method 1	Method 2	Method 1	Method 2
Number of CpG islands detected	5,211	522	195,706	26,876
Average length of island (bp)	390	535	395	497
Percent of sequence predicted as CpG	5.9	0.8	2.6	0.4
Percent of first exons that overlap a CpG island	44	25	42	22
Percent of first exons with first position of exon contained inside a CpG island	37	22	40	21
Average distance between first exon and closest CpG island (bp)	1,013	10,486	2,182	17,021
Expected distance between first exon and closest CpG island (bp)	3,262	32,567	7,164	55,811

Table 14. Distribution of repetitive DNA in the compartmentalized shotgun assembly sequence.

Repetitive elements	Megabases in assembled sequences	Percent of assembly	Previously predicted (%) (83)
Alu	288	9.9	10.0
Mammalian interspersed repeat (MIR)	66	2.3	1.7
Medium reiteration (MER)	50	1.7	1.6
Long terminal repeat (LTR)	155	5.3	5.6
Long interspersed nucleotide element (LINE)	466	16.1	16.7
Total	1025	35.3	35.6

terminated to be in the same family and the same complete Lek cluster (essentially paralogous genes) (89). Initially, each chromosome was represented as a string of genes ordered by the start codons for predicted genes along the chromosome. We considered the two strands as a single string, because local inversions are relatively common events relative to large-scale duplications. Each gene was indexed according to the protein family and Lek complete cluster (89). All pairs of indexed gene strings were then aligned in both the forward and reverse directions with the Smith-Waterman algorithm (90). A match between two proteins of the same Lek complete cluster was given a score of 10 and a mismatch -10, with gap open and extend penalties of -4 and -1. With these parameters, 19 conserved interchromosomal blocks of duplication were observed, all of which were also detected and expanded by the comprehensive method described below. The detection of only a relatively small number of block duplications was a consequence of using an intrinsically conservative method grounded in the conservative constraints of the complete Lek clusters.

In the second, more comprehensive approach, we aligned all chromosomes directly with one another using an algorithm based on the MUMmer system (91). This alignment method uses a suffix tree data structure and a linear-time algorithm to align long sequences very rapidly; for example, two chromosomes of 100 Mbp can be aligned in less than 20 min (on a Compaq Alpha computer) with 4 gigabytes of memory. This procedure was used recently to identify numerous large-scale segmental duplications among the five chromosomes of *A. thaliana* (92); in that organism, the method revealed that 60% of the genome (66 Mbp) is covered by 24 very large duplicated segments. For *Arabidopsis*, a DNA-based alignment was sufficient to reveal the segmental duplications between chromosomes; in the human genome, DNA alignments at the whole-chromosome level are insufficiently sensitive. Therefore, a modified procedure was developed and applied, as follows. First, all 26,588 proteins (9,675,713 million amino acids) were concatenated end-to-end in order as they occur along each of the 24 chromosomes, irrespective of strand location. The concatenated protein set was then aligned against each chromosome by the MUMmer algorithm. The resulting matches were clustered to extract all sets of three or more protein matches that occur in close proximity on two different chromosomes (93); these represent the candidate segmental duplications. A series of filters were developed and applied to remove likely false-positives from this set; for example, small blocks that were spread across many proteins were removed. To refine the

clustering methods, a shuffled protein set was first created by taking the 26,588 proteins, randomizing their order, and then partitioning them into 24 shuffled chromosomes, each containing the same number of proteins as the true genome. This shuffled protein set has the identical composition to the real genome; in particular, every protein and every domain appears the same number of times. The complete algorithm was then applied to both the real and the shuffled data, with the results on the shuffled data being used to estimate the false-positive rate. The algorithm after filtering yielded 10,310 gene pairs in 1077 duplicated blocks containing 3522 distinct genes; tandemly duplicated expansions in many of the blocks explain the excess of gene pairs to distinct genes. In the shuffled data, by contrast, only 370 gene pairs were found, giving a false-positive estimate of 3.6%. The most likely explanation for the 1077 block duplications is ancient segmental duplications. In many cases, the order of the proteins has been shuffled, although proximity is preserved. Out of the 1077 blocks, 159 contain only three genes, 137 contain four genes, and 781 contain five or more genes.

To illustrate the extent of the detected duplications, Fig. 13 shows all 1077 block duplications indexed to each chromosome in 24 panels in which only duplications mapped to the indexed chromosome are displayed. The figure makes it clear that the duplications are ubiquitous in the genome. One feature that it displays is many relatively small chromosomal stretches, with one-to-many duplication relationships that are graphically striking. One such example captured by the analysis is the well-documented olfactory receptor (OR) family, which is scattered in blocks throughout the genome and which has been analyzed for genome-deployment reconstruc-

tion several evolutionary stages (94). The figure also illustrates that some chromosomes, such as chromosome 2, contain many more detected large-scale duplications than others. Indeed, one of the largest duplicated segments is a large block of 33 proteins on chromosome 2, spread among eight smaller blocks in 2p, that aligns to a paralogous set on chromosome 14, with one rearrangement (see chromosomes 2 and 14 panels in Fig. 13). The proteins are not contiguous but span a region containing 97 proteins on chromosome 2 and 332 proteins on chromosome 14. The likelihood of observing this many duplicated proteins by chance, even over a span of this length, is 2.3×10^{-68} (93). This duplicated set spans 20 Mbp on chromosome 2 and 63 Mbp on chromosome 14, over 70% of the latter chromosome. Chromosome 2 also contains a block duplication that is nearly as large, which is shared by chromosome arm 2q and chromosome 12. This duplication incorporates two of the four known Hox gene clusters, but considerably expands the extent of the duplications proximally and distally on the pair of chromosome arms. This breadth of duplication is also seen on the two chromosomes carrying the other two Hox clusters.

An additional large duplication, between chromosomes 18 and 20, serves as a good example to illustrate some of the features common to many of the other observed large duplications (Fig. 13, inset). This duplication contains 64 detected ordered intrachromosomal pairs of homologous genes. After discounting a 40-Mb stretch of chromosome 18 free of matches to chromosome 20, which is likely to represent a large insert (between the gene assignments "Krup rel" and "collagen rel" on chromosome 18 in Fig. 13), the full duplication segment covers 36 Mb on chromosome 18 and 28 Mb on chromosome 20.

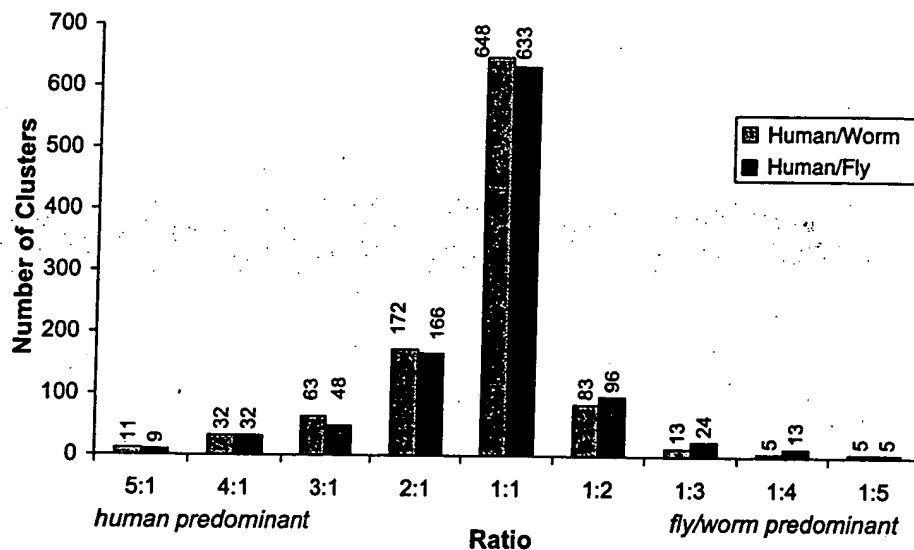


Fig. 12. Gene duplication in complete protein clusters. The predicted protein sets of human, worm, and fly were subjected to Lek clustering (27). The numbers of clusters with varying ratios (whole number) of human versus worm and human versus fly proteins per cluster were plotted.

By this measure, the duplication segment spans nearly half of each chromosome's net length. The most likely scenario is that the whole span of this region was duplicated as a single very large block, followed by shuffling owing to smaller scale rearrangements. As such, at least four subsequent rearrangements would need to be invoked to explain the relative insertions and inversions seen in the duplicated segment interval. The 64 protein pairs in this alignment occur among 217 protein assignments on chromosome 18, and among 322 protein assignments on chromosome 20, for a density of involved proteins of 20 to 30%. This is consistent with an ancient large-scale duplication followed by subsequent gene loss on one or both chromosomes. Loss of just one member of a gene pair subsequent to the duplication would result in a failure to score a gene pair in the block; less than 50% gene loss on the chromosomes would lead to the duplication density observed here. As an independent verification of the significance of the alignments detected, it can be seen that a substantial number of the pairs of aligning proteins in this duplication, including some of those annotated (Fig. 13), are those populating small Lek complete clusters (see above). This indicates that they are members of very small families of paralogs; their relative scarcity within the genome validates the uniqueness and robust nature of their alignments.

Two additional qualitative features were observed among many of the large-scale duplications. First, several proteins with disease associations, with OMIM (Online Mendelian Inheritance in Man) assignments, are members of duplicated segments (see web table 2 on *Science* Online at www.sciencemag.org/cgi/content/full/291/5507/1304/DC1). We have also observed a few instances where paralogs on both duplicated segments are associated with similar disease conditions. Notable among these genes are proteins involved in hemostasis (coagulation factors) that are associated with bleeding disorders, transcriptional regulators like the homeobox proteins associated with developmental disorders, and potassium channels associated with cardiovascular conduction abnormalities. For each of these disease genes, closer study of the paralogous genes in the duplicated segment may reveal new insights into disease causation, with further investigation needed to determine whether they might be involved in the same or similar genetic diseases. Second, although there is a conserved number of proteins and coding exons predicted for specific large duplicated spans within the chromosome 18 to 20 alignment, the genomic DNA of chromosome 18 in these specific spans is in some cases more than 10-fold longer than the corresponding chromosome 20 DNA. This selective accretion of noncoding DNA (or conversely, loss of noncoding DNA) on one of a

pair of duplicated chromosome regions was observed in many compared regions. Hypotheses to explain which mechanisms foster these processes must be tested.

Evaluation of the alignment results gives some perspective on dating of the duplications. As noted above, large-scale ancient segmental duplication in fact best explains many of the blocks detected by this genome-wide analysis. The regions of human chromosomes involved in the large-scale duplications expanded upon above (chromosomes 2 to 14, 2 to 12, and 18 to 20) are each syntenic to a distinct mouse chromosomal region. The corresponding mouse chromosomal regions are much more similar in sequence conservation, and even in order, to their human synteny partners than the human duplication regions are to each other. Further, the corresponding mouse chromosomal regions each bear a significant proportion of genes orthologous to the human genes on which the human duplication assignments were made. On the basis of these factors, the corresponding mouse chromosomal spans, at coarse resolution, appear to be products of the same large-scale duplications observed in humans. Although further detailed analysis must be carried out once a more complete genome is assembled for mouse, the underlying large duplications appear to predate the two species' divergence. This dates the duplications, at the latest, before divergence of the primate and rodent lineages. This date can be further refined upon examination of the synteny between human chromosomes and those of chicken, pufferfish (*Fugu rubripes*), or zebrafish (95). The only substantial syntenic stretches mapped in these species corresponding to both pairs of human duplications are restricted to the Hox cluster regions. When the synteny of these regions (or others) to human chromosomes is extended with further mapping, the ages of the nearly chromosome-length duplications seen in humans are likely to be dated to the root of vertebrate divergence.

The MUMmer-based results demonstrate large block duplications that range in size from a few genes to segments covering most of a chromosome. The extent of segmental duplications raises the question of whether an ancient whole-genome duplication event is the underlying explanation for the numerous duplicated regions (96). The duplications have undergone many deletions and subsequent rearrangements; these events make it difficult to distinguish between a whole-genome duplication and multiple smaller events. Further analysis, focused especially on comparing the estimated ages of all the block duplications, derived partially from interspecies genome comparisons, will be necessary to determine which of these two hypotheses is more likely. Comparisons of genomes of different vertebrates, and even cross-phyla genome comparisons, will allow for the deconvolution of duplications to eventually re-

veal the stagewise history of our genome, and with it a history of the emergence of many of the key functions that distinguish us from other living things.

6 A Genome-Wide Examination of Sequence Variations

Summary. Computational methods were used to identify single-nucleotide polymorphisms (SNPs) by comparison of the Celera sequence to other SNP resources. The SNP rate between two chromosomes was ~1 per 1200 to 1500 bp. SNPs are distributed nonrandomly throughout the genome. Only a very small proportion of all SNPs (<1%) potentially impact protein function based on the functional analysis of SNPs that affect the predicted coding regions. This results in an estimate that only thousands, not millions, of genetic variations may contribute to the structural diversity of human proteins.

Having a complete genome sequence enables researchers to achieve a dramatic acceleration in the rate of gene discovery, but only through analysis of sequence variation in DNA can we discover the genetic basis for variation in health among human beings. Whole-genome shotgun sequencing is a particularly effective method for detecting sequence variation in tandem with whole-genome assembly. In addition, we compared the distribution and attributes of SNPs ascertained by three other methods: (i) alignment of the Celera consensus sequence to the PFP assembly, (ii) overlap of high-quality reads of genomic sequence (referred to as "Kwok"; 1,120,195 SNPs) (97), and (iii) reduced representation shotgun sequencing (referred to as "TSC"; 632,640 SNPs) (98). These data were consistent in showing an overall nucleotide diversity of $\sim 8 \times 10^{-4}$, marked heterogeneity across the genome in SNP density, and an overwhelming preponderance of noncoding variation that produces no change in expressed proteins.

6.1 SNPs found by aligning the Celera consensus to the PFP assembly

Ideally, methods of SNP discovery make full use of sequence depth and quality at every site, and quantitatively control the rate of false-positive and false-negative calls with an explicit sampling model (99). Comparison of consensus sequences in the absence of these details necessitated a more ad hoc approach (quality scores could not readily be obtained for the PFP assembly). First, all sequence differences between the two consensus sequences were identified; these were then filtered to reduce the contribution of sequencing errors and misassembly. As a measure of the effectiveness of the filtering step, we monitored the ratio of transition and transversion substitutions, because a 2:1 ratio has been well documented as typical in mammalian evolution (100) and in human SNPs

(101, 102). The filtering steps consisted of removing variants where the quality score in the Celera consensus was less than 30 and where the density of variants was greater than 5 in 400 bp. These filters resulted in shifting the transition-to-transversion ratio from 1.57:1 to 1.89:1. When applied to 2.3 Gbp of alignments between the Celera and PFP consensus sequences, these filters resulted in identification of 2,104,820 putative SNPs from a total of 2,778,474 substitution differences. Overlaps between this set of SNPs and those found by other methods are described below.

6.2 Comparisons to public SNP databases

Additional SNPs, including 2,536,021 from dbSNP (www.ncbi.nlm.nih.gov/SNP) and 13,150 from HGMD (Human Gene Mutation Database, from the University of Wales, UK), were mapped on the Celera consensus sequence by a sequence similarity search with the program PowerBlast (103). The two largest data sets in dbSNP are the Kwok and TSC sets, with 47% and 25% of the dbSNP records. Low-quality alignments with partial coverage of the dbSNP sequence and alignments that had less than 98% sequence identity between the Celera sequence and the dbSNP flanking sequence were eliminated. dbSNP sequences mapping to multiple locations on the Celera genome were discarded. A total of 2,336,935 dbSNP variants were mapped to 1,223,038 unique locations on the Celera sequence, implying considerable redundancy in dbSNP. SNPs in the TSC set mapped to 585,811 unique genomic locations, and SNPs in the Kwok set mapped to 438,032 unique locations. The combined unique SNPs counts used in this analysis, including Celera-PFP, TSC, and Kwok, is 2,737,668. Table 15 shows that a substantial fraction of SNPs identified by one of these methods was also found by another method. The very high overlap (36.2%) between the Kwok and Celera-PFP SNPs may be due in part to the use by Kwok of sequences that went into the PFP assembly. The unusually low overlap (16.4%) between the Kwok and TSC sets is due

Table 15. Overlap of SNPs from genome-wide SNP databases. Table entries are SNP counts for each pair of data sets. Numbers in parentheses are the fraction of overlap, calculated as the count of overlapping SNPs divided by the number of SNPs in the smaller of the two databases compared. Total SNP counts for the databases are: Celera-PFP, 2,104,820; TSC, 585,811; and Kwok 438,032. Only unique SNPs in the TSC and Kwok data sets were included.

	TSC	Kwok
Celera-PFP	188,694 (0.322)	158,532 (0.362)
TSC		72,024 (0.164)

their being the smallest two sets. In addition, 24.5% of the Celera-PFP SNPs overlap with SNPs derived from the Celera genome sequences (46). SNP validation in population samples is an expensive and laborious process, so confirmation on multiple data sets may provide an efficient initial validation "in silico" (by computational analysis).

One means of assessing whether the three sets of SNPs provide the same picture of human variation is to tally the frequencies of the six possible base changes in each set of SNPs (Table 16). Previous measures of nucleotide diversity were mostly derived from small-scale analysis on candidate genes (101), and our analysis with all three data sets validates the previous observations at the whole-genome scale. There is remarkable homogeneity between the SNPs found in the Kwok set, the TSC set, and in our whole-genome shotgun (46) in this substitution pattern. Compared with the rest of the data sets, Celera-PFP deviates slightly from the 2:1 transition-to-transversion ratio observed in the other SNP sets. This result is not unexpected, because some fraction of the computationally identified SNPs in the Celera-PFP comparison may in fact be sequence errors. A 2:1 transition:transversion ratio for the bona fide SNPs would be obtained if one assumed that 15% of the sequence differences in the Celera-PFP set were a result of (presumably random) sequence errors.

6.3 Estimation of nucleotide diversity from ascertained SNPs

The number of SNPs identified varied widely across chromosomes. In order to normalize these values to the chromosome size and sequence coverage, we used π , the standard statistic for nucleotide diversity (104). Nucleotide diversity is a measure of per-site heterozygosity, quantifying the probability that a pair of chromosomes drawn from the population will differ at a nucleotide site. In order to calculate nucleotide diversity for each chromosome, we need to know the number of nucleotide sites that were surveyed for variation, and in methods like reduced representation sequencing, we need to know the sequence quality and the depth of coverage at each

site. These data are not readily available, so we could not estimate nucleotide diversity from the TSC effort. Estimation of nucleotide diversity from high-quality sequence overlaps should be possible, but again, more information is needed on the details of all the alignments.

Estimation of nucleotide diversity from a shotgun assembly entails calculating for each column of the multialignment, the probability that two or more distinct alleles are present, and the probability of detecting a SNP if in fact the alleles have different sequence (i.e., the probability of correct sequence calls). The greater the depth of coverage and the higher the sequence quality, the higher is the chance of successfully detecting a SNP (105). Even after correcting for variation in coverage, the nucleotide diversity appeared to vary across autosomes. The significance of this heterogeneity was tested by analysis of variance, with estimates of π for 100-kbp windows to estimate variability within chromosomes (for the Celera-PFP comparison, $F = 29.73$, $P < 0.0001$).

Average diversity for the autosomes estimated from the Celera-PFP comparison was 8.94×10^{-4} . Nucleotide diversity on the X chromosome was 6.54×10^{-4} . The X is expected to be less variable than autosomes, because for every four copies of autosomes in the population, there are only three X chromosomes, and this smaller effective population size means that random drift will more rapidly remove variation from the X (106).

Having ascertained nucleotide variation genome-wide, it appears that previous estimates of nucleotide diversity in humans based on samples of genes were reasonably accurate (101, 102, 106, 107). Genome-wide, our estimate of nucleotide diversity was 8.98×10^{-4} for the Celera-PFP alignment, and a published estimate averaged over 10 densely resequenced human genes was 8.00×10^{-4} (108).

6.4 Variation in nucleotide diversity across the human genome

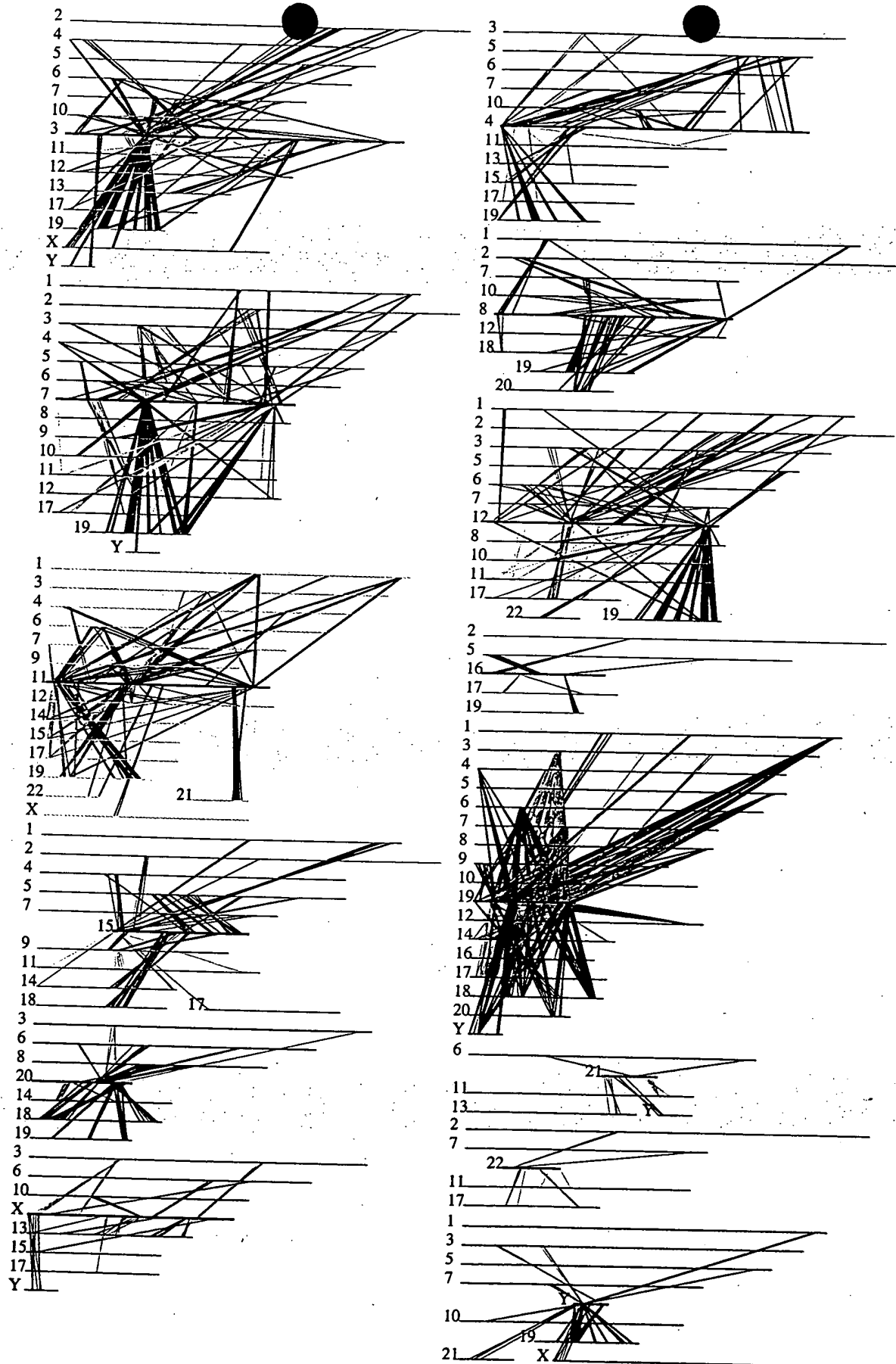
Such an apparently high degree of variability among chromosomes in SNP density raises the question of whether there is heterogeneity at a finer scale within chromo-

Table 16. Summary of nucleotide changes in different SNP data sets.

SNP data set	A/G (%)	C/T (%)	A/C (%)	A/T (%)	C/G (%)	T/G (%)	Transition: transversion
Celera-PFP	30.7	30.7	10.3	8.6	9.2	10.3	1.59:1
Kwok*	33.7	33.8	8.5	7.0	8.6	8.4	2.07:1
TSC†	33.3	33.4	8.8	7.3	8.6	8.6	1.99:1

*November 2000 release of the NCBI database dbSNP (www.ncbi.nlm.nih.gov/SNP/) with the method defined as Overlap SnpDetectionWithPolyBayes. The submitter of the data is Pui-Yan Kwok from Washington University. †November 2000 release of NCBI dbSNP (www.ncbi.nlm.nih.gov/SNP/) with the methods defined as TSC-Sanger, TSC-WICGR, and TSC-WUGSC. The submitter of the data is Lincoln Stein from Cold Spring Harbor Laboratory.





somes, and whether this heterogeneity is greater than expected by chance. If SNPs occur by random and independent mutations, then it would seem that there ought to be a Poisson distribution of numbers of SNPs in fragments of arbitrary constant size. The observed dispersion in the distribution of SNPs in 100-kbp fragments was far greater than predicted from a Poisson distribution (Fig. 14). However, this simplistic model ignores the different recombination rates and population histories that exist in different regions of the genome. Population genetics theory holds that we can account for this variation with a mathematical formulation called the neutral coalescent (109). Applying well-tested algorithms for simulating the neutral coalescent with recombination (110), and using an effective population size of 10,000 and a per-base recombination rate equal to the mutation rate (111), we generated a distribution of numbers of SNPs by this model as well (112). The observed distribution of SNPs has a much larger variance than either the Poisson model or the coalescent model, and the difference is highly significant. This implies that there is significant variability across the genome in SNP density, an observation that begs an explanation.

Several attributes of the DNA sequence may affect the local density of SNPs, including the rate at which DNA polymerase makes errors and the efficacy of mismatch repair. One key factor that is likely to be associated with SNP density is the G+C content, in part because methylated cytosines in CpG dinucleotides tend to undergo deamination to form thymine, accounting for a nearly 10-fold increase in the mutation rate of CpGs over other dinucle-

otides. We tallied the GC content and nucleotide diversities in 100-kbp windows across the entire genome and found that the correlation between them was positive ($r = 0.21$) and highly significant ($P < 0.0001$), but G+C content accounted for only a small part of the variation.

6.5 SNPs by genomic class

To test homogeneity of SNP densities across functional classes, we partitioned sites into intergenic (defined as >5 kbp from any predicted transcription unit), 5'-UTR, exonic (missense and silent), intronic, and 3'-UTR for 10,239 known genes, derived from the NCBI RefSeq database and all human genes predicted from the Celera Otto annotation. In coding regions, SNPs were categorized as either silent, for those that do not change amino acid sequence, or missense, for those that change the protein product. The ratio of missense to silent coding SNPs in Celera-PFP, TSC, and Kwok sets (1.12, 0.91, and 0.78, respectively) shows a markedly reduced frequency of missense variants compared with the neutral expectation, consistent with the elimination by natural selection of a fraction of the deleterious amino acid changes (112). These ratios are comparable to the missense-to-silent ratios of 0.88 and 1.17 found by Cargill *et al.* (101) and by Halushka *et al.* (102). Similar results were observed in SNPs derived from Celera shotgun sequences (46).

It is striking how small is the fraction of SNPs that lead to potentially dysfunctional alterations in proteins. In the 10,239 RefSeq genes, missense SNPs were only about

0.12, 0.14, and 0.17% of the total SNP counts in Celera-PFP, TSC, and Kwok SNPs, respectively. Nonconservative protein changes constitute an even smaller fraction of missense SNPs (47, 41, and 40% in Celera-PFP, Kwok, and TSC). Intergenic regions have been virtually unstudied (113), and we note that 75% of the SNPs we identified were intergenic (Table 17). The SNP rate was highest in introns and lowest in exons. The SNP rate was lower in intergenic regions than in introns, providing one of the first discriminators between these two classes of DNA. These SNP rates were confirmed in the Celera SNPs, which also exhibited a lower rate in exons than in introns, and in extragenic regions than in introns (46). Many of these intergenic SNPs will provide valuable information in the form of markers for linkage and association studies, and some fraction is likely to have a regulatory function as well.

7 An Overview of the Predicted Protein-Coding Genes in the Human Genome

Summary. This section provides an initial computational analysis of the predicted protein set with the aim of cataloging prominent differences and similarities when the human genome is compared with other fully sequenced eukaryotic genomes. Over 40% of the predicted protein set in humans cannot be ascribed a molecular function by methods that assign proteins to known families. A protein domain-based analysis provides a detailed catalog of the prominent differences in the human genome when compared with the fly and worm genomes. Prominent among these are domain expansions in proteins involved in developmental regulation and in cellular processes such as neuronal function, hemostasis, acquired immune response, and cytoskeletal complexity. The final enumeration of protein families and details of protein structure will rely on additional experimental work and comprehensive manual curation.

A preliminary analysis of the predicted human protein-coding genes was conducted. Two methods were used to analyze and classify the molecular functions of 26,588 predicted proteins that represent 26,383 gene predictions with at least two lines of evidence as described above. The first method was based on an analysis at the level of protein families, with both the publicly available Pfam database (114, 115) and Celera's Panther Classification (CPC) (Fig. 15) (116). The second method was based on an analysis at the level of protein domains, with both the Pfam and SMART databases (115, 117).

The results presented here are preliminary and are subject to several limitations.

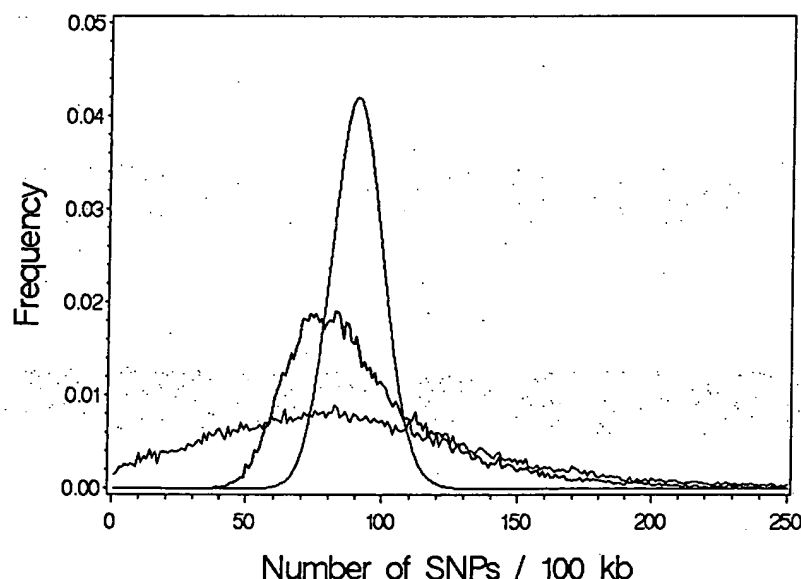


Fig. 14. SNP density in each 100-kbp interval as determined with Celera-PFP SNPs. The color codes are as follows: black, Celera-PFP SNP density; blue, coalescent model; and red, Poisson distribution. The figure shows that the distribution of SNPs along the genome is nonrandom and is not entirely accounted for by a coalescent model of regional history.

Both the gene predictions and functional assignments have been made by using computational tools, although the statistical models in Panther, Pfam, and SMART have been built, annotated, and reviewed by expert biologists. In the set of computationally predicted genes, we expect both false-positive predictions (some of these may in fact be inactive pseudogenes) and false-negative predictions (some human genes will not be computationally predicted). We also expect errors in delimiting the boundaries of exons and genes. Similarly, in the automatic functional assignments, we also expect both false-positive and false-negative predictions. The functional assignment protocol focuses on protein families that tend to be found across several organisms, or on families of known human genes. Therefore, we do not assign a function to many genes that are not in large families, even if the function is known. Unless otherwise specified, all enumeration of the genes in any given family or functional category was taken from the set of 26,588 predicted proteins, which were assigned functions by using statistical score cutoffs defined for models in Panther, Pfam, and SMART.

For this initial examination of the predicted human protein set, three broad questions were asked: (i) What are the likely molecular functions of the predicted gene products, and how are these proteins categorized with current classification methods? (ii) What are the core functions that appear to be common across the animals?

(iii) How does the human protein complement differ from that of other sequenced eukaryotes?

7.1 Molecular functions of predicted human proteins

Figure 15 shows an overview of the putative molecular functions of the predicted 26,588 human proteins that have at least two lines of supporting evidence. About 41% (12,809) of the gene products could not be classified from this initial analysis and are termed proteins with unknown functions. Because our automatic classification methods treat only relatively large protein families, there are a number of "unclassified" sequences that do, in fact, have a known or predicted function. For the 60% of the protein set that have automatic functional predictions, the specific protein functions have been placed into broad classes. We focus here on molecular function (rather than higher order cellular processes) in order to classify as many proteins as possible. These functional predictions are based on similarity to sequences of known function.

In our analysis of the 12,731 additional low-confidence predicted genes (those with only one piece of supporting evidence), only 636 (5%) of these additional putative genes were assigned molecular functions by the automated methods. One-third of these 636 predicted genes represented endogenous retroviral proteins, further suggesting that the majority of

these unknown-function genes are not real genes. Given that most of these additional 12,095 genes appear to be unique among the genomes sequenced to date, many may simply represent false-positive gene predictions.

The most common molecular functions are the transcription factors and those involved in nucleic acid metabolism (nucleic acid enzyme). Other functions that are highly represented in the human genome are the receptors, kinases, and hydrolases. Not surprisingly, most of the hydrolases are proteases. There are also many proteins that are members of proto-oncogene families, as well as families of "select regulatory molecules": (i) proteins involved in specific steps of signal transduction such as heterotrimeric GTP-binding proteins (G proteins) and cell cycle regulators; and (ii) proteins that modulate the activity of kinases, G proteins, and phosphatases.

Table 17. Distribution of SNPs in classes of genomic regions.

Genomic region class	Size of region examined (Mb)	Celera-PF SNP density (SNP/Mb)
Intergenic	2185	707
Gene (intron + exon)	646	917
Intron	615	921
First intron	164	808
Exon	31	529
First exon	10	592

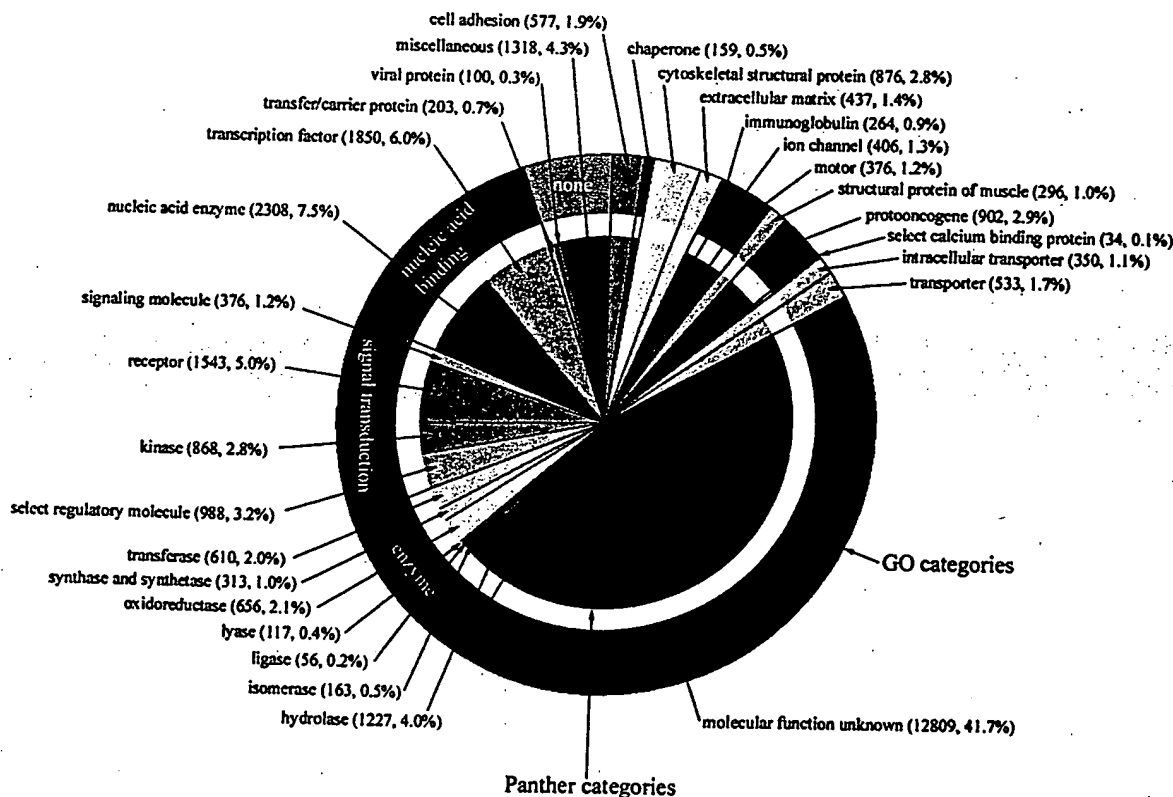


Fig. 15. Distribution of the molecular functions of 26,383 human genes. Each slice lists the numbers and percentages (in parentheses) of human gene functions assigned to a given category of molecular function. The outer circle shows the assignment to molecular function categories in the Gene Ontology (GO) (179), and the inner circle shows the assignment to Celera's Panther molecular function categories (116).

7.2 Evolutionary conservation of core processes

Because of the various "model organism" genome-sequencing projects that have already been completed, reasonable comparative information is available for beginning the analysis of the evolution of the human genome. The genomes of *S. cerevisiae* ("bakers' yeast") (118) and two diverse invertebrates, *C. elegans* (a nematode worm) (119) and *D. melanogaster* (fly) (26), as well as the first plant genome, *A. thaliana*, recently completed (92), provide a diverse background for genome comparisons.

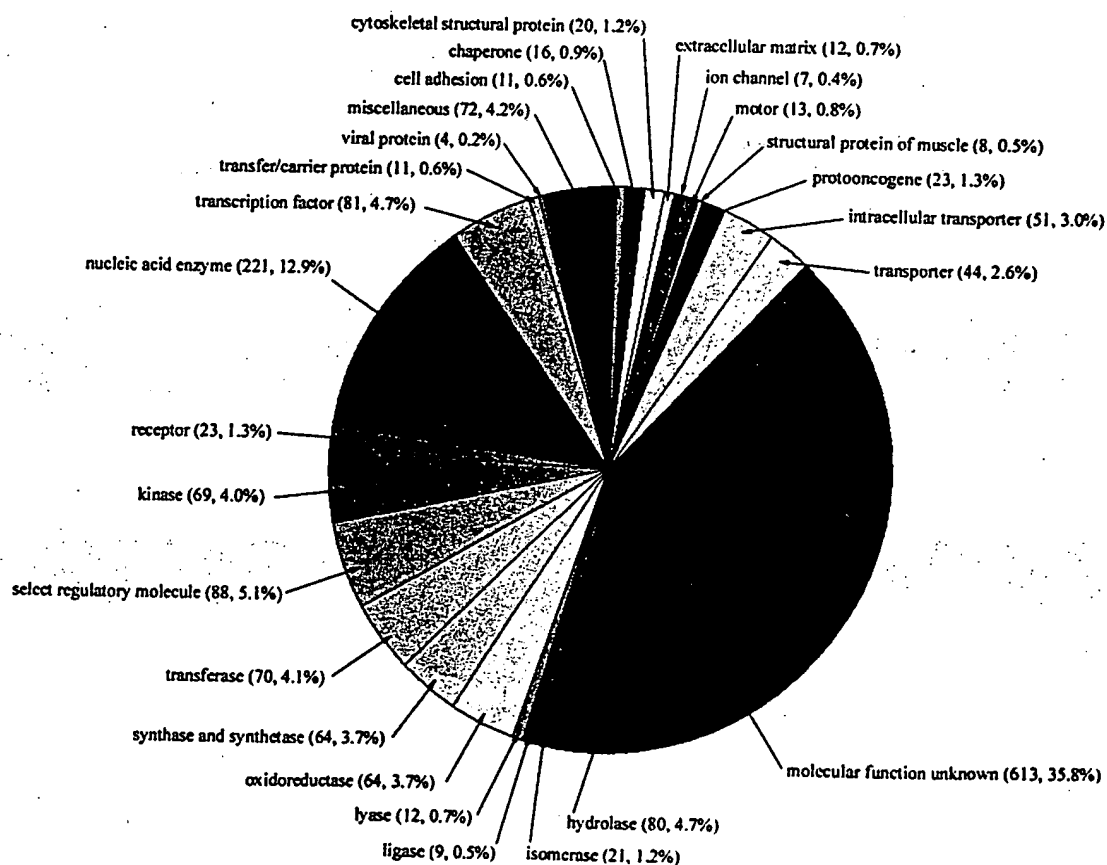
We enumerated the "strict orthologs" conserved between human and fly, and between human and worm (Fig. 16) to address the question, What are the core functions that appear to be common across the animals? The concept of orthology is important because if two genes are orthologs, they can be traced by descent to the common ancestor of the two organisms (an "evolutionarily conserved protein set"), and therefore are likely to perform similar conserved functions in the different organisms. It is critical in this analysis to separate orthologs (a gene that appears in two organisms by descent from a common ancestor) from paralogs (a gene that appears in more than one copy in a given organism by a duplication event) because paralogs may subsequently diverge in function. Following the yeast-worm ortholog comparison in

(120), we identified two different cases for each pairwise comparison (human-fly and human-worm). The first case was a pair of genes, one from each organism, for which there was no other close homolog in either organism. These are straightforwardly identified as orthologous, because there are no additional members of the families that complicate separating orthologs from paralogs. The second case is a family of genes with more than one member in either or both of the organisms being compared. Chervitz *et al.* (120) deal with this case by analyzing a phylogenetic tree that described the relationships between all of the sequences in both organisms, and then looked for pairs of genes that were nearest neighbors in the tree. If the nearest-neighbor pairs were from different organisms, those genes were presumed to be orthologs. We note that these nearest neighbors can often be confidently identified from pairwise sequence comparison without having to examine a phylogenetic tree (see legend to Fig. 16). If the nearest neighbors are not from different organisms, there has been a paralogous expansion in one or both organisms after the speciation event (and/or a gene loss by one organism). When this one-to-one correspondence is lost, defining an ortholog becomes ambiguous. For our initial computational overview of the predicted human protein set, we could not answer this question for every predicted protein. Therefore, we con-

sider only "strict orthologs," i.e., the proteins with unambiguous one-to-one relationships (Fig. 16). By these criteria, there are 2758 strict human-fly orthologs, 2031 human-worm (1523 in common between these sets). We define the evolutionarily conserved set as those 1523 human proteins that have strict orthologs in both *D. melanogaster* and *C. elegans*.

The distribution of the functions of the conserved protein set is shown in Fig. 16. Comparison with Fig. 15 shows that, not surprisingly, the set of conserved proteins is not distributed among molecular functions in the same way as the whole human protein set. Compared with the whole human set (Fig. 15), there are several categories that are overrepresented in the conserved set by a factor of ~2 or more. The first category is nucleic acid enzymes, primarily the transcriptional machinery (notably DNA/RNA methyltransferases, DNA/RNA polymerases, helicases, DNA ligases, DNA- and RNA-processing factors, nucleases, and ribosomal proteins). The basic transcriptional and translational machinery is well known to have been conserved over evolution, from bacteria through to the most complex eukaryotes. Many ribonucleoproteins involved in RNA splicing also appear to be conserved among the animals. Other enzyme types are also overrepresented (transferases, oxidoreductases, ligases, lyases, and isomerases). Many of these en-

Fig. 16. Functions of putative orthologs across vertebrate and invertebrate genomes. Each slice lists the number and percentages (in parentheses) of "strict orthologs" between the human, fly, and worm genomes involved in a given category of molecular function. "Strict orthologs" are defined here as bi-directional BLAST best hits (180) such that each orthologous pair (i) has a BLASTP *P*-value of $\leq 10^{-10}$ (120), and (ii) has a more significant BLASTP score than any paralogs in either organism, i.e., there has likely been no duplication subsequent to speciation that might make the orthology ambiguous. This measure is quite strict and is a lower bound on the number of orthologs. By these criteria, there are 2758 strict human-fly orthologs, and 2031 human-worm orthologs (1523 in common between these sets).



zymes are involved in intermediary metabolism. The only exception is the hydrolase category, which is not significantly overrepresented in the shared protein set. Proteases form the largest part of this category, and several large protease families have expanded in each of these three organisms after their divergence. The category of select regulatory molecules is also overrepresented in the conserved set. The major conserved families are small guanosine triphosphatases (GTPases) (especially the Ras-related superfamily, including ADP ribosylation factor) and cell cycle regulators (particularly the cullin family, cyclin C family, and several cell division protein kinases). The last two significantly overrepresented categories are protein transport and trafficking, and chaperones. The most conserved groups in these categories are proteins involved in coated vesicle-mediated transport, and chaperones involved in protein folding and heat-shock response [particularly the DNAJ family, and heat-shock protein 60 (HSP60), HSP70, and HSP90 families]. These observations provide only a conservative estimate of the protein families in the context of specific cellular processes that were likely derived from the last common ancestor of the human, fly, and worm. As stated before, this analysis does not provide a complete estimate of conservation across the three animal genomes, as paralogous duplication makes the determination of true orthologs difficult within the members of conserved protein families.

7.3 Differences between the human genome and other sequenced eukaryotic genomes

To explore the molecular building blocks of the vertebrate taxon, we have compared the human genome with the other sequenced eukaryotic genomes at three levels: molecular functions, protein families, and protein domains.

Molecular differences can be correlated with phenotypic differences to begin to reveal the developmental and cellular processes that are unique to the vertebrates. Tables 18 and 19 display a comparison among all sequenced eukaryotic genomes, over selected protein/domain families (defined by sequence similarity, e.g., the serine-threonine protein kinases) and superfamilies (defined by shared molecular function, which may include several sequence-related families, e.g., the cytochromes). In these tables we have focused on (super) families that are either very large or that differ significantly in humans compared with the other sequenced eukaryotic genomes. We have found that the most prominent human expansions are in proteins involved in (i) acquired immune functions; (ii) neural development, structure, and functions; (iii) intercellular and intracellular signaling pathways

in development and homeostasis; (iv) hemostasis; and (v) apoptosis.

Acquired immunity. One of the most striking differences between the human genome and the *Drosophila* or *C. elegans* genome is the appearance of genes involved in acquired immunity (Tables 18 and 19). This is expected, because the acquired immune response is a defense system that only occurs in vertebrates. We observe 22 class I and 22 class II major histocompatibility complex (MHC) antigen genes and 114 other immunoglobulin genes in the human genome. In addition, there are 59 genes in the cognate immunoglobulin receptor family. At the domain level, this is exemplified by an expansion and recruitment of the ancient immunoglobulin fold to constitute molecules such as MHC, and of the integrin fold to form several of the cell adhesion molecules that mediate interactions between immune effector cells and the extracellular matrix. Vertebrate-specific proteins include the paracrine immune regulators family of secreted 4- α helical bundle proteins, namely the cytokines and chemokines. Some of the cytoplasmic signal transduction components associated with cytokine receptor signal transduction are also features that are poorly represented in the fly and worm. These include protein domains found in the signal transducer and activator of transcription (STATs), the suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STATs (PIAS). In contrast, many of the animal-specific protein domains that play a role in innate immune response, such as the Toll receptors, do not appear to be significantly expanded in the human genome.

Neural development, structure, and function. In the human genome, as compared with the worm and fly genomes, there is a marked increase in the number of members of protein families that are involved in neural development. Examples include neurotrophic factors such as ependymin, nerve growth factor, and signaling molecules such as semaphorins, as well as the number of proteins involved directly in neural structure and function such as myelin proteins, voltage-gated ion channels, and synaptic proteins such as synaptotagmin. These observations correlate well with the known phenotypic differences between the nervous systems of these taxa, notably (i) the increase in the number and connectivity of neurons; (ii) the increase in number of distinct neural cell types (as many as a thousand or more in human compared with a few hundred in fly and worm) (121); (iii) the increased length of individual axons; and (iv) the significant increase in glial cell number, especially the appearance of myelinating glial cells, which are electrically inert supporting cells differentiated from the same stem cells as neurons. A number

of prominent protein expansions are involved in the processes of neural development. Of the extracellular domains that mediate cell adhesion, the connexin domain-containing proteins (122) exist only in humans. These proteins, which are not present in the *Drosophila* or *C. elegans* genomes, appear to provide the constitutive subunits of intercellular channels and the structural basis for electrical coupling. Pathway finding by axons and neuronal network formation is mediated through a subset of ephrins and their cognate receptor tyrosine kinases that act as positional labels to establish topographical projections (123). The probable biological role for the semaphorins (22 in human compared with 6 in the fly and 2 in the worm) and their receptors (neuropilins and plexins) is that of axonal guidance molecules (124). Signaling molecules such as neurotrophic factors and some cytokines have been shown to regulate neuronal cell survival, proliferation, and axon guidance (125). Notch receptors and ligands play important roles in glial cell fate determination and gliogenesis (126).

Other human expanded gene families play key roles directly in neural structure and function. One example is synaptotagmin (expanded more than twofold in humans relative to the invertebrates), originally found to regulate synaptic transmission by serving as a Ca^{2+} sensor (or receptor) during synaptic vesicle fusion and release (127). Of interest is the increased co-occurrence in humans of PDZ and the SH3 domains in neuronal-specific adaptor molecules; examples include proteins that likely modulate channel activity at synaptic junctions (128). We also noted expansions in several ion-channel families (Table 19), including the EAG subfamily (related to cyclic nucleotide-gated channels), the voltage-gated calcium/sodium channel family, the inward-rectifier potassium channel family, and the voltage-gated potassium channel, α subunit family. Voltage-gated sodium and potassium channels are involved in the generation of action potentials in neurons. Together with voltage-gated calcium channels, they also play a key role in coupling action potentials to neurotransmitter release, in the development of neurites, and in short-term memory. The recent observation of a calcium-regulated association between sodium channels and synaptotagmin may have consequences for the establishment and regulation of neuronal excitability (129).

Myelin basic protein and myelin-associated glycoprotein are major classes of protein components in both the central and peripheral nervous system of vertebrates. Myelin P0 is a major component of peripheral myelin, and myelin proteolipid and myelin oligodendrocyte glycoprotein are found in the central nervous system. Mutations in any of these

THE HUMAN GENOME

Table 18. Domain-based comparative analysis of proteins in *H. sapiens* (H), *D. melanogaster* (F), *C. elegans* (W), *S. cerevisiae* (Y), and *A. thaliana* (A). The predicted protein set of each of the above eukaryotic organisms was analyzed with Pfam version 5.5 using E value cutoffs of 0.001. The number of proteins containing the specified Pfam domains as well as the total number of domains (in parentheses) are shown in each column. Domains were categorized into cellular processes for presentation. Some domains (i.e., SH2) are listed in

more than one cellular process. Results of the Pfam analysis may differ from results obtained based on human curation of protein families, owing to the limitations of large-scale automatic classifications. Representative examples of domains with reduced counts owing to the stringent E value cutoff used for this analysis are marked with a double asterisk (**). Examples include short divergent and predominantly alpha-helical domains, and certain classes of cysteine-rich zinc finger proteins.

Accession number	Domain name	Domain description	H	F	W	Y	A
<i>Developmental and homeostatic regulators</i>							
PF02039	Adrenomedullin	Adrenomedullin	1	0	0	0	0
PF00212	ANP	Atrial natriuretic peptide	2	0	0	0	0
PF00028	Cadherin	Cadherin domain	100 (550)	14 (157)	16 (66)	0	0
PF00214	Calc_CGRP_IAPP	Calcitonin/CGRP/IAPP family	3	0	0	0	0
PF01110	CNTF	Ciliary neurotrophic factor	1	0	0	0	0
PF01093	Clusterin	Clusterin	3	0	0	0	0
PF00029	Connexin	Connexin	14 (16)	0	0	0	0
PF00976	ACTH_domain	Corticotropin ACTH domain	1	0	0	0	0
PF00473	CRF	Corticotropin-releasing factor family	2	1	0	0	0
PF00007	Cys_knot	Cystine-knot domain	10 (11)	2	0	0	0
PF00778	DIX	Dix domain	5	2	4	0	0
PF00322	Endothelin	Endothelin family	3	0	0	0	0
PF00812	Ephrin	Ephrin	7 (8)	2	4	0	0
PF01404	EPH_lbd	Ephrin receptor ligand binding domain	12	2	1	0	0
PF00167	FGF	Fibroblast growth factor	23	1	1	0	0
PF01534	Frizzled	Frizzled/Smoothened family membrane region	9	7	3	0	0
PF00236	Hormone6	Glycoprotein hormones	1	0	0	0	0
PF01153	Glypican	Glypican	14	2	1	0	0
PF01271	Granin	Granin (chromogranin or secretogranin)	3	0	0	0	0
PF02058	Guanylin	Guanylin precursor	1	0	0	0	0
PF00049	Insulin	Insulin/IGF/Relaxin family	7	4	0	0	0
PF00219	IGFBP	Insulin-like growth factor binding proteins	10	0	0	0	0
PF02024	Leptin	Leptin	1	0	0	0	0
PF00193	Xlink	LINK (hyaluron binding)	13 (23)	0	1	0	0
PF00243	NGF	Nerve growth factor family	3	0	0	0	0
PF02158	Neuregulin	Neuregulin family	4	0	0	0	0
PF00184	Hormone5	Neurohypophysial hormones	1	0	0	0	0
PF02070	NMU	Neuromedin U	1	0	0	0	0
PF00066	Notch	Notch (DSL) domain	3 (5)	2 (4)	2 (6)	0	0
PF00865	Osteopontin	Osteopontin	1	0	0	0	0
PF00159	Hormone3	Pancreatic hormone peptides	3	0	0	0	0
PF01279	Parathyroid	Parathyroid hormone family	2	0	0	0	0
PF00123	Hormone2	Peptide hormone	5 (9)	0	0	0	0
PF00341	PDGF	Platelet-derived growth factor (PDGF)	5	1	0	0	0
PF01403	Sema	Sema domain	27 (29)	8 (10)	3 (4)	0	0
PF01033	Somatomedin_B	Somatomedin B domain	5 (8)	3	0	0	0
PF00103	Hormone	Somatotropin	1	0	0	0	0
PF02208	Sorb	Sorbin homologous domain	2	0	0	0	0
PF02404	SCF	Stem cell factor	2	0	0	0	0
PF01034	Syndecan	Syndecan domain	3	1	1	0	0
PF00020	TNFR_c6	TNFR/NGFR cysteine-rich region	17 (31)	1	0	0	0
PF00019	TGF-β	Transforming growth factor β-like domain	27 (28)	6	4	0	0
PF01099	Uteroglobulin	Uteroglobulin family	3	0	0	0	0
PF01160	Opioids_neuropep	Vertebrate endogenous opioids neuropeptide	3	0	0	0	0
PF00110	Wnt	Wnt family of developmental signaling proteins	18	7 (10)	5	0	0
<i>Hemostasis</i>							
PF01821	ANATO	Anaphylotoxin-like domain	6 (14)	0	0	0	0
PF00386	C1q	C1q domain	24	0	0	0	0
PF00200	Disintegrin	Disintegrin	18	2	3	0	0
PF00754	F5_F8_type_C	F5/8 type C domain	15 (20)	5 (6)	2	0	0
PF01410	COLFI	Fibrillar collagen C-terminal domain	10	0	0	0	0
PF00039	Fn1	Fibronectin type I domain	5 (18)	0	0	0	0
PF00040	Fn2	Fibronectin type II domain	11 (16)	0	0	0	0
PF00051	Kringle	Kringle domain	15 (24)	2	2	0	0
PF01823	MACPF	MAC/Perforin domain	6	0	0	0	0
PF00354	Pentaxin	Pentaxin family	9	0	0	0	0
PF00277	SAA_proteins	Serum amyloid A protein	4	0	0	0	0
PF00084	Sushi	Sushi domain (SCR repeat)	53 (191)	11 (42)	8 (45)	0	0
PF02210	TSPN	Thrombospondin N-terminal-like domains	14	1	0	0	0
PF01108	Tissue_fac	Tissue factor	1	0	0	0	0
PF00868	Transglutamin_N	Transglutaminase family	6	1	0	0	0
PF00927	Transglutamin_C	Transglutaminase family	8	1	0	0	0

Table 18 (Continued)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00594	Gla	Vitamin K-dependent carboxylation/gamma-carboxyglutamic (GLA) domain	11	0	0	0	0
<i>Immune response</i>							
PF00711	Defensin_beta	Beta defensin	1	0	0	0	0
PF00748	Calpain_inhib	Calpain inhibitor repeat	3 (9)	0	0	0	0
PF00666	Cathelicidins	Cathelicidins	2	0	0	0	0
PF00129	MHC_I	Class I histocompatibility antigen, domains alpha 1 and 2	18 (20)	0	0	0	0
PF00993	MHC_II_alpha**	Class II histocompatibility antigen, alpha domain	5 (6)	0	0	0	0
PF00969	MHC_II_beta**	Class II histocompatibility antigen, beta domain	7	0	0	0	0
PF00879	Defensin_propep	Defensin propeptide	3	0	0	0	0
PF01109	GM-CSF	Granulocyte-macrophage colony-stimulating factor	1	0	0	0	0
PF00047	Ig	Immunoglobulin domain	381 (930)	125 (291)	67 (323)	0	0
PF00143	Interferon	Interferon alpha/beta domain	7 (9)	0	0	0	0
PF00714	IFN-gamma	Interferon gamma	1	0	0	0	0
PF00726	IL10	Interleukin-10	1	0	0	0	0
PF02372	IL15	Interleukin-15	1	0	0	0	0
PF00715	IL2	Interleukin-2	1	0	0	0	0
PF00727	IL4	Interleukin-4	1	0	0	0	0
PF02025	IL5	Interleukin-5	1	0	0	0	0
PF01415	IL7	Interleukin-7/9 family	1	0	0	0	0
PF00340	IL1	Interleukin-1	7	0	0	0	0
PF02394	IL1_propep	Interleukin-1 propeptide	1	0	0	0	0
PF02059	IL3	Interleukin-3	1	0	0	0	0
PF00489	IL6	Interleukin-6/G-CSF/MGF family	2	0	0	0	0
PF01291	LIF_OSM	Leukemia inhibitory factor (LIF)/oncostatin (OSM) family	2	0	0	0	0
PF00323	Defensins	Mammalian defensin	2	0	0	0	0
PF01091	PTN_MK	PTN/MK heparin-binding protein	2	0	0	0	0
PF00277	SAA_proteins	Serum amyloid A protein	4	0	0	0	0
PF00048	IL8	Small cytokines (intercrine/chemokine), interleukin-8 like	32	0	0	0	0
PF01582	TIR	TIR domain	18	8	2	0	131 (143)
PF00229	TNF	TNF (tumor necrosis factor) family	12	0	0	0	0
PF00088	Trefoil	Trefoil (P-type) domain	5 (6)	0	2	0	0
<i>PI-PY-rho GTPase signaling</i>							
PF00779	BTK	BTK motif	5	1	0	0	0
PF00168	C2	C2 domain	73 (101)	32 (44)	24 (35)	6 (9)	66 (90)
PF00609	DAGKa	Diacylglycerol kinase accessory domain (presumed)	9	4	7	0	6
PF00781	DAGKc	Diacylglycerol kinase catalytic domain (presumed)	10	8	8	2	11 (12)
PF00610	DEP	Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP)	12 (13)	4	10	5	2
PF01363	FYVE	FYVE zinc finger	28 (30)	14	15	5	15
PF00996	GDI	GDP dissociation inhibitor	6	2	1	1	3
PF00503	G-alpha	G-protein alpha subunit	27 (30)	10	20 (23)	2	5
PF00631	G-gamma	G-protein gamma like domains	16	5	5	1	0
PF00616	RasGAP	GTPase-activator protein for Ras-like GTPase	11	5	8	3	0
PF00618	RasGEFN	Guanine nucleotide exchange factor for Ras-like GTPases; N-terminal motif	9	2	3	5	0
PF00625	Guanylate_kin	Guanylate kinase	12	8	7	1	4
PF02189	ITAM	Immunoreceptor tyrosine-based activation motif	3	0	0	0	0
PF00169	PH	PH domain	193 (212)	72 (78)	65 (68)	24	23
PF00130	DAG_PE-bind	Phorbol esters/diacylglycerol binding domain (C1 domain)	45 (56)	25 (31)	26 (40)	1 (2)	4
PF00388	PI-PLC-X	Phosphatidylinositol-specific phospholipase C, X domain	12	3	7	1	8
PF00387	PI-PLC-Y	Phosphatidylinositol-specific phospholipase C, Y domain	11	2	7	1	8
PF00640	PID	Phosphotyrosine interaction domain (PTB/PID)	24 (27)	13	11 (12)	0	0
PF02192	PI3K_p85B	PI3-kinase family, p85-binding domain	2	1	1	0	0
PF00794	PI3K_rbd	PI3-kinase family, ras-binding domain	6	3	1	0	0
PF01412	ArfGAP	Putative GTP-ase activating protein for Arf	16	9	8	6	15
PF02196	RBD	Raf-like Ras-binding domain	6 (7)	4	1	0	0
PF02145	Rap_GAP	Rap/ran-GAP	5	4	2	0	0
PF00788	RA	Ras association (RalGDS/AF-6) domain	18 (19)	7 (9)	6	1	0
PF00071	Ras	Ras family	126	56 (57)	51	23	78
PF00617	RasGEF	RasGEF domain	21	8	7	5	0
PF00615	RGS	Regulator of G protein signaling domain	27	6 (7)	12 (13)	1	0
PF02197	Rila	Regulatory subunit of type II PKA R-subunit	4	1	2	1	0

THE HUMAN GENOME

Table 18 (Continued)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00620	RhoGAP	RhoGAP domain	59	19	20	9	8
PF00621	RhoGEF	RhoGEF domain	46	23 (24)	18 (19)	3	0
PF00536	SAM	SAM domain (Sterile alpha motif)	29 (31)	15	8	3	6
PF01369	Sec7	Sec7 domain	13	5	5	5	9
PF00017	SH2	Src homology 2 (SH2) domain	87 (95)	33 (39)	44 (48)	1	3
PF00018	SH3	Src homology 3 (SH3) domain	143 (182)	55 (75)	46 (61)	23 (27)	4
PF01017	STAT	STAT protein	7	1	1 (2)	0	0
PF00790	VHS	VHS domain	4	2	4	4	8
PF00568	WH1	WH1 domain	7	2	2 (3)	1	0
<i>Domains involved in apoptosis</i>							
PF00452	Bcl-2	Bcl-2	9	2	1	0	0
PF02180	BH4	Bcl-2 homology region 4	3	0	1	0	0
PF00619	CARD	Caspase recruitment domain	16	0	2	0	0
PF00531	Death	Death domain	16	5	7	0	0
PF01335	DED	Death effector domain	4 (5)	0	0	0	0
PF02179	BAG	Domain present in Hsp70 regulators	5 (8)	3	2	1	5
PF00656	ICE_p20	ICE-like protease (caspase) p20 domain	11	7	3	0	0
PF00653	BIR	Inhibitor of Apoptosis domain	8 (14)	5 (9)	2 (3)	1 (2)	0
<i>Cytoskeletal</i>							
PF00022	Actin	Actin	61 (64)	15 (16)	12	9 (11)	24
PF00191	Annexin	Annexin	16 (55)	4 (16)	4 (11)	0	6 (16)
PF00402	Calponin	Calponin family	13 (22)	3	7 (19)	0	0
PF00373	Band_41	FERM domain (Band 4.1 family)	29 (30)	17 (19)	11 (14)	0	0
PF00880	Nebulin_repeat	Nebulin repeat	4 (148)	1 (2)	1	0	0
PF00681	Plectin_repeat	Plectin repeat	2 (11)	0	0	0	0
PF00435	Spectrin	Spectrin repeat	31 (195)	13 (171)	10 (93)	0	0
PF00418	Tubulin-binding	Tau and MAP proteins, tubulin-binding	4 (12)	1 (4)	2 (8)	0	0
PF00992	Troponin	Troponin	4	6	8	0	0
PF02209	VHP	Villin headpiece domain	5	2	2	0	5
PF01044	Vinculin	Vinculin family	4	2	1	0	0
<i>ECM adhesion</i>							
PF01391	Collagen	Collagen triple helix repeat (20 copies)	65 (279)	10 (46)	174 (384)	0	0
PF01413	C4	C-terminal tandem repeated domain in type 4 procollagen	6 (11)	2 (4)	3 (6)	0	0
PF00431	CUB	CUB domain	47 (69)	9 (47)	43 (67)	0	0
PF00008	EGF	EGF-like domain	108 (420)	45 (186)	54 (157)	0	1
PF00147	Fibrinogen_C	Fibrinogen beta and gamma chains, C-terminal globular domain	26	10 (11)	6	0	0
PF00041	Fn3	Fibronectin type III domain	106 (545)	42 (168)	34 (156)	0	1
PF00757	Furin-like	Furin-like cysteine rich region	5	2	1	0	0
PF00357	Integrin_A	Integrin alpha cytoplasmic region	3	1	2	0	0
PF00362	Integrin_B	Integrins, beta chain	8	2	2	0	0
PF00052	Laminin_B	Laminin B (Domain IV)	8 (12)	4 (7)	6 (10)	0	0
PF00053	Laminin_EGF	Laminin EGF-like (Domains III and V)	24 (126)	9 (62)	11 (65)	0	0
PF00054	Laminin_G	Laminin G domain	30 (57)	18 (42)	14 (26)	0	0
PF00055	Laminin_Nterm	Laminin N-terminal (Domain VI)	10	6	4	0	0
PF00059	Lectin_c	Lectin C-type domain	47 (76)	23 (24)	91 (132)	0	0
PF01463	LRRCT	Leucine rich repeat C-terminal domain	69 (81)	23 (30)	7 (9)	0	0
PF01462	LRRNT	Leucine rich repeat N-terminal domain	40 (44)	7 (13)	3 (6)	0	0
PF00057	Ldl_recept_a	Low-density lipoprotein receptor domain class A	35 (127)	33 (152)	27 (113)	0	0
PF00058	Ldl_recept_b	Low-density lipoprotein receptor repeat class B	15 (96)	9 (56)	7 (22)	0	0
PF00530	SRCR	Scavenger receptor cysteine-rich domain	11 (46)	4 (8)	1 (2)	0	0
PF00084	Sushi	Sushi domain (SCR repeat)	53 (191)	11 (42)	8 (45)	0	0
PF00090	Tsp_1	Thrombospondin type 1 domain	41 (66)	11 (23)	18 (47)	0	0
PF00092	Vwa	von Willebrand factor type A domain	34 (58)	0	17 (19)	0	1
PF00093	Vwc	von Willebrand factor type C domain	19 (28)	6 (11)	2 (5)	0	0
PF00094	Vwd	von Willebrand factor type D domain	15 (35)	3 (7)	9	0	0
<i>Protein interaction domains</i>							
PF00244	14-3-3	14-3-3 proteins	20	3	3	2	15
PF00023	Ank	Ank repeat	145 (404)	72 (269)	75 (223)	12 (20)	66 (111)
PF00514	Armadillo_seg	Armadillo/beta-catenin-like repeats	22 (56)	11 (38)	3 (11)	2 (10)	25 (67)
PF00168	C2	C2 domain	73 (101)	32 (44)	24 (35)	6 (9)	66 (90)
PF00027	cNMP_binding	Cyclic nucleotide-binding domain	26 (31)	21 (33)	15 (20)	2 (3)	22
PF01556	DnaJ_C	DnaJ C terminal region	12	9	5	3	19
PF00226	DnaJ	DnaJ domain	44	34	33	20	93
PF00036	EFhand**	EF hand	83 (151)	64 (117)	41 (86)	4 (11)	120 (328)
PF00611	FCH	Fes/CIP4 homology domain	9	3	2	4	0
PF01846	FF	FF domain	4 (11)	4 (10)	3 (16)	2 (5)	4 (8)
PF00498	FHA	FHA domain	13	15	7	13 (14)	17

myelin proteins result in severe demyelination, which is a pathological condition in which the myelin is lost and the nerve conduction is severely impaired (130). Humans have at least 10 genes belonging to four different families involved in myelin produc-

tion (five myelin P0, three myelin proteolipid, myelin basic protein, and myelin-oligodendrocyte glycoprotein, or MOG), and possibly more-remotely related members of the MOG family. Flies have only a single myelin proteolipid, and worms have none at all.

Intercellular and intracellular signaling pathways in development and homeostasis. Many protein families that have expanded in humans relative to the invertebrates are involved in signaling processes, particularly in response to development and differentiation

Table 18 (Continued)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00254	FKBP	FKBP-type peptidyl-prolyl cis-trans isomerases	15 (20)	7 (8)	7 (13)	4	24 (29)
PF01590	GAF	GAF domain	7 (8)	2 (4)	1	0	10
PF01344	Kelch	Kelch motif	54 (157)	12 (48)	13 (41)	3	102 (178)
PF00560	LRR**	Leucine Rich Repeat	25 (30)	24 (30)	7 (11)	1	15 (16)
PF00917	MATH	MATH domain	11	5	88 (161)	1	61 (74)
PF00989	PAS	PAS domain	18 (19)	9 (10)	6	1	13 (18)
PF00595	PDZ	PDZ domain (Also known as DHR or GLGF)	96 (154)	60 (87)	46 (66)	2	5
PF00169	PH	PH domain	193 (212)	72 (78)	65 (68)	24	23
PF01535	PPR**	PPR repeat	5	3 (4)	0	1	474 (2485)
PF00536	SAM	SAM domain (Sterile alpha motif)	29 (31)	15	8	3	6
PF01369	Sec7	Sec7 domain	13	5	5	5	9
PF00017	SH2	Src homology 2 (SH2) domain	87 (95)	33 (39)	44 (48)	1	3
PF00018	SH3	Src homology 3 (SH3) domain	143 (182)	55 (75)	46 (61)	23 (27)	4
PF01740	STAS	STAS domain	5	1	6	2	13
PF00515	TPR**	TPR domain	72 (131)	39 (101)	28 (54)	16 (31)	65 (124)
PF00400	WD40**	WD40 domain	136 (305)	98 (226)	72 (153)	56 (121)	167 (344)
PF00397	WW	WW domain	32 (53)	24 (39)	16 (24)	5 (8)	11 (15)
PF00569	ZZ	ZZ-Zinc finger present in dystrophin, CBP/p300	10 (11)	13	10	2	10
<i>Nuclear interaction domains</i>							
PF01754	Zf-A20	A20-like zinc finger	2 (8)	2	2	0	8
PF01388	ARID	ARID DNA binding domain	11	6	4	2	7
PF01426	BAH	BAH domain	8 (10)	7 (8)	4 (5)	5	21 (25)
PF00643	Zf-B_box**	B-box zinc finger	32 (35)	1	2	0	0
PF00533	BRCT	BRCA1 C Terminus (BRCT) domain	17 (28)	10 (18)	23 (35)	10 (16)	12 (16)
PF00439	Bromodomain	Bromodomain	37 (48)	16 (22)	18 (26)	10 (15)	28
PF00651	BTB	BTB/POZ domain	97 (98)	62 (64)	86 (91)	1 (2)	30 (31)
PF00145	DNA_methylase	C-5 cytosine-specific DNA methylase	3 (4)	1	0	0	13 (15)
PF00385	Chromo	chromo' (CHR)romatin Organization MODifier domain	24 (27)	14 (15)	17 (18)	1 (2)	12
PF00125	Histone	Core histone H2A/H2B/H3/H4	75 (81)	5	71 (73)	8	48
PF00134	Cyclin	Cyclin	19	10	10	11	35
PF00270	DEAD	DEAD/DEAH box helicase	63 (66)	48 (50)	55 (57)	50 (52)	84 (87)
PF01529	Zf-DHHC	DHHC zinc finger domain	15	20	16	7	22
PF00646	F-box**	F-box domain	16	15	309 (324)	9	165 (167)
PF00250	Fork_head	Fork head domain	35 (36)	20 (21)	15	4	0
PF00320	GATA	GATA zinc finger	11 (17)	5 (6)	8 (10)	9	26
PF01585	G-patch	G-patch domain	18	16	13	4	14 (15)
PF00010	HLH**	Helix-loop-helix DNA-binding domain	60 (61)	44	24	4	39
PF00850	Hist_deacetyl	Histone deacetylase family	12	5 (6)	8 (10)	5	10
PF00046	Homeobox	Homeobox domain	160 (178)	100 (103)	82 (84)	6	66
PF01833	TIG	IPT/TIG domain	29 (53)	11 (13)	5 (7)	2	1
PF02373	JmjC	JmjC domain	10	4	6	4	7
PF02375	JmjN	JmjN domain	7	4	2	3	7
PF00013	KH-domain	KH domain	28 (67)	14 (32)	17 (46)	4 (14)	27 (61)
PF01352	KRAB	KRAB box	204 (243)	0	0	0	0
PF00104	Hormone_rec	Ligand-binding domain of nuclear hormone receptor	47	17	142 (147)	0	0
PF00412	LIM	LIM domain containing proteins	62 (129)	33 (83)	33 (79)	4 (7)	10 (16)
PF00917	MATH	MATH domain	11	5	88 (161)	1	61 (74)
PF00249	Myb_DNA-binding	Myb-like DNA-binding domain	32 (43)	18 (24)	17 (24)	15 (20)	243 (401)
PF02344	Myc-LZ	Myc leucine zipper domain	1	0	0	0	0
PF01753	Zf-MYND	MYND finger	14	14	9	1	7
PF00628	PHD	PHD-finger	68 (86)	40 (53)	32 (44)	14 (15)	96 (105)
PF00157	Pou	Pou domain—N-terminal to homeobox domain	15	5	4	0	0
PF02257	RFX_DNA_binding	RFX DNA-binding domain	7	2	1	1	0
PF00076	Rrm	RNA recognition motif (a.k.a. RRM, RBD, or RNP domain)	224 (324)	127 (199)	94 (145)	43 (73)	232 (369)
PF02037	SAP	SAP domain	15	8	5	5	6 (7)
PF00622	SPRY	SPRY domain	44 (51)	10 (12)	5 (7)	3	6
PF01852	START	START domain	10	2	6	0	23
PF00907	T-box	T-box	17 (19)	8	22	0	0

Table 18 (Continued)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF02135	Zf-TAZ	TAZ finger	2 (3)	1 (2)	6 (7)	0	10 (15)
PF01285	TEA	TEA domain	4	1	1	1	0
PF02176	Zf-TRAF	TRAF-type zinc finger	6 (9)	1 (3)	1	0	2
PF00352	TBP	Transcription factor TFIID (or TATA-binding protein, TBP)	2 (4)	4 (8)	2 (4)	1 (2)	2 (4)
PF00567	TUDOR	TUDOR domain	9 (24)	9 (19)	4 (5)	0	2
PF00642	Zf-CCCH	Zinc finger, C-x8-C-x5-C-x3-H type (and similar)	17 (22)	6 (8)	22 (42)	3 (5)	31 (46)
PF00096	Zf-C2H2**	Zinc finger, C2H2 type	564 (4500)	234 (771)	68 (155)	34 (56)	21 (24)
PF00097	Zf-C3HC4	Zinc finger, C3HC4 type (RING finger)	135 (137)	57	88 (89)	18	298 (304)
PF00098	Zf-CCHC	Zinc knuckle	9 (17)	6 (10)	17 (33)	7 (13)	68 (91)

(Tables 18 and 19). They include secreted hormones and growth factors, receptors, intracellular signaling molecules, and transcription factors.

Developmental signaling molecules that are enriched in the human genome include growth factors such as wnt, transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), nerve growth factor, platelet derived growth factor (PDGF), and ephrins. These growth factors affect tissue differentiation and a wide range of cellular processes involving actin-cytoskeletal and nuclear regulation. The corresponding receptors of these developmental ligands are also expanded in humans. For example, our analysis suggests at least 8 human ephrin genes (2 in the fly, 4 in the worm) and 12 ephrin receptors (2 in the fly, 1 in the worm). In the wnt signaling pathway, we find 18 wnt family genes (6 in the fly, 5 in the worm) and 12 frizzled receptors (6 in the fly, 5 in the worm). The Groucho family of transcriptional corepressors downstream in the wnt pathway are even more markedly expanded, with 13 predicted members in humans (2 in the fly, 1 in the worm).

Extracellular adhesion molecules involved in signaling are expanded in the human genome (Tables 18 and 19). The interactions of several of these adhesion domains with extracellular matrix proteoglycans play a critical role in host defense, morphogenesis, and tissue repair (131). Consistent with the well-defined role of heparan sulfate proteoglycans in modulating these interactions (132), we observe an expansion of the heparin sulfate sulfotransferases in the human genome relative to worm and fly. These sulfotransferases modulate tissue differentiation (133). A similar expansion in humans is noted in structural proteins that constitute the actin-cytoskeletal architecture. Compared with the fly and worm, we observe an explosive expansion of the nebulin (35 domains per protein on average), aggrecan (12 domains per protein on average), and plectin (5 domains per protein on average) repeats in humans. These repeats are present in proteins involved in modulating the actin-cytoskeleton with predominant expression in neuronal, muscle, and vascular tissues.

Comparison across the five sequenced eukaryotic organisms revealed several expanded protein families and domains involved in cytoplasmic signal transduction (Table 18). In particular, signal transduction pathways playing roles in developmental regulation and acquired immunity were substantially enriched. There is a factor of 2 or greater expansion in humans in the Ras superfamily GTPases and the GTPase activator and GTP exchange factors associated with them. Although there are about the same number of tyrosine kinases in the human and *C. elegans* genomes, in humans there is an increase in the SH2, PTB, and ITAM domains involved in phosphotyrosine signal transduction. Further, there is a twofold expansion of phosphodiesterases in the human genome compared with either the worm or fly genomes.

The downstream effectors of the intracellular signaling molecules include the transcription factors that transduce developmental fates. Significant expansions are noted in the ligand-binding nuclear hormone receptor class of transcription factors compared with the fly genome, although not to the extent observed in the worm (Tables 18 and 19). Perhaps the most striking expansion in humans is in the C2H2 zinc finger transcription factors. Pfam detects a total of 4500 C2H2 zinc finger domains in 564 human proteins, compared with 771 in 234 fly proteins. This means that there has been a dramatic expansion not only in the number of C2H2 transcription factors, but also in the number of these DNA-binding motifs per transcription factor (8 on average in humans, 3.3 on average in the fly, and 2.3 on average in the worm). Furthermore, many of these transcription factors contain either the KRAB or SCAN domains, which are not found in the fly or worm genomes. These domains are involved in the oligomerization of transcription factors and increase the combinatorial partnering of these factors. In general, most of the transcription factor domains are shared between the three animal genomes, but the reassortment of these domains results in organism-specific transcription factor families. The domain combinations found in the human, fly, and worm include the BTB with C2H2 in the fly and humans, and

homeodomains alone or in combination with Pou and LIM domains in all of the animal genomes. In plants, however, a different set of transcription factors are expanded, namely, the myb family, and a unique set that includes VP1 and AP2 domain-containing proteins (134). The yeast genome has a paucity of transcription factors compared with the multicellular eukaryotes, and its repertoire is limited to the expansion of the yeast-specific C6 transcription factor family involved in metabolic regulation.

While we have illustrated expansions in a subset of signal transduction molecules in the human genome compared with the other eukaryotic genomes, it should be noted that most of the protein domains are highly conserved. An interesting observation is that worms and humans have approximately the same number of both tyrosine kinases and serine/threonine kinases (Table 19). It is important to note, however, that these are merely counts of the catalytic domain; the proteins that contain these domains also display a wide repertoire of interaction domains with significant combinatorial diversity.

Hemostasis. Hemostasis is regulated primarily by plasma proteases of the coagulation pathway and by the interactions that occur between the vascular endothelium and platelets. Consistent with known anatomical and physiological differences between vertebrates and invertebrates, extracellular adhesion domains that constitute proteins integral to hemostasis are expanded in the human relative to the fly and worm (Tables 18 and 19). We note the evolution of domains such as FIMAC, FN1, FN2, and C1q that mediate surface interactions between hematopoietic cells and the vascular matrix. In addition, there has been extensive recruitment of more-ancient animal-specific domains such as VWA, VWC, VWD, kringle, and FN3 into multidomain proteins that are involved in hemostatic regulation. Although we do not find a large expansion in the total number of serine proteases, this enzymatic domain has been specifically recruited into several of these multidomain proteins for proteolytic regulation in the vascular compartment. These are represented in plasma proteins that belong to the kinin and complement pathways. There is a

significant expansion in two families of matrix metalloproteases: ADAM (a disintegrin and metalloprotease) and MMPs (matrix metalloproteases) (Table 19). Proteolysis of extracellular matrix (ECM) proteins is critical for tissue development and for tissue degradation in diseases such as cancer, arthritis, Alzheimer's disease, and a variety of inflammatory conditions (135, 136). ADAMs are a family of integral membrane proteins with a pivotal role in fibrinogenolysis and modulating interactions between hematopoietic components and the vascular matrix components. These proteins have been shown to cleave matrix proteins, and even signaling molecules: ADAM-17 converts tumor necrosis factor- α , and ADAM-10 has been implicated in the Notch signaling pathway (135). We have identified 19 members of the matrix metalloprotease family, and a total of 51 members of the ADAM and ADAM-TS families.

Apoptosis. Evolutionary conservation of some of the apoptotic pathway components across eukarya is consistent with its central role in developmental regulation and as a response to pathogens and stress signals. The signal transduction pathways involved in programmed cell death, or apoptosis, are mediated by interactions between well-characterized domains that include extracellular domains, adaptor (protein-protein interaction) domains, and those found in effector and regulatory enzymes (137). We enumerated the protein counts of central adaptor and effector enzyme domains that are found only in the apoptotic pathways to provide an estimate of divergence across eukarya and relative expansion in the human genome when compared with the fly and worm (Table 18). Adaptor domains found in proteins restricted only to apoptotic regulation such as the DED domains are vertebrate-specific, whereas others like BIR, CARD, and Bcl2 are represented in the fly and worm (although the number of Bcl2 family members in humans is significantly expanded). Although plants and yeast lack the caspases, caspase-like molecules, namely the para- and meta-caspases, have been reported in these organisms (138). Compared with other animal genomes, the human genome shows an expansion in the adaptor and effector domain-containing proteins involved in apoptosis, as well as in the proteases involved in the cascade such as the caspase and calpain families.

Expansions of other protein families.
Metabolic enzymes. There are fewer cytochrome P450 genes in humans than in either the fly or worm. Lipoygenases (six in humans), on the other hand, appear to be specific to the vertebrates and plants, whereas the lipoygenase-activating proteins (four in humans) may be vertebrate-specific. Lipoygenases are involved in arachidonic acid metabolism, and they and their activators have been implicated

in diverse human pathology ranging from allergic responses to cancers. One of the most surprising human expansions, however, is in the number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (46 in humans, 3 in the fly, and 4 in the worm). There is, however, evidence for many retrotrans-

posed GAPDH pseudogenes (139), which may account for this apparent expansion. However, it is interesting that GAPDH, long known as a conserved enzyme involved in basic metabolism found across all phyla from bacteria to humans, has recently been shown to have other functions. It has a second cat-

Table 19. Number of proteins assigned to selected Panther families or subfamilies in *H. sapiens* (H), *D. melanogaster* (F), *C. elegans* (W), *S. cerevisiae* (Y), and *A. thaliana* (A).

Panther family/subfamily*	H	F	W	Y	A
<i>Neural structure, function, development</i>					
Ependymin	1	0	0	0	0
Ion channels	17	12	56	0	0
Acetylcholine receptor	11	24	27	0	0
Amiloride-sensitive/degenerin	22	9	9	0	30
CNG/EAG	16	3	3	0	0
IRK	10	2	4	0	0
ITP/ryanodine	61	51	59	0	19
Neurotransmitter-gated	10	0	0	0	0
P2X purinoceptor	12	12	48	1	5
TASK	15	3	3	1	0
Transient receptor	22	4	8	2	2
Voltage-gated Ca ²⁺ alpha	10	3	2	0	0
Voltage-gated Ca ²⁺ alpha-2	5	2	2	0	0
Voltage-gated Ca ²⁺ beta	1	0	0	0	0
Voltage-gated Ca ²⁺ gamma	33	5	11	0	0
Voltage-gated K ⁺ alpha	6	2	3	0	0
Voltage-gated KQT	11	4	4	9	1
Voltage-gated Na ⁺	1	0	0	0	0
Myelin basic protein	5	0	0	0	0
Myelin PO	3	1	0	0	0
Myelin proteolipid	1	0	0	0	0
Myelin-oligodendrocyte glycoprotein	2	0	0	0	0
Neuropilin	9	2	0	0	0
Plexin	22	6	2	0	0
Semaphorin	10	3	3	0	0
Synaptotagmin	<i>Immune response</i>				
Defensin	3	0	0	0	0
Cytokine†	86	14	1	0	0
GCSF	1	0	0	0	0
GMCSF	1	0	0	0	0
Intercrine alpha	15	0	0	0	0
Intercrine beta	5	0	0	0	0
Inteferon	8	0	0	0	0
Interleukin	26	1	1	0	0
Leukemia inhibitory factor	1	0	0	0	0
MCSF	1	0	0	0	0
Peptidoglycan recognition protein	2	13	0	0	0
Pre-B cell enhancing factor	1	0	0	0	0
Small inducible cytokine A	14	0	0	0	0
Sl cytokine	2	0	0	0	0
TNF	9	0	0	0	0
Cytokine receptor†	62	1	0	0	0
Bradykinin/C-C chemokine receptor	7	0	0	0	0
Fl cytokine receptor	2	0	0	0	0
Interferon receptor	3	0	0	0	0
Interleukin receptor	32	0	0	0	0
Leukocyte tyrosine kinase receptor	3	0	0	0	0
MCSF receptor	1	0	0	0	0
TNF receptor	3	0	0	0	0
Immunoglobulin receptor†	59	0	0	0	0
T-cell receptor alpha chain	16	0	0	0	0
T-cell receptor beta chain	15	0	0	0	0
T-cell receptor gamma chain	1	0	0	0	0
T-cell receptor delta chain	1	0	0	0	0
Immunoglobulin FC receptor	8	0	0	0	0
Killer cell receptor	16	0	0	0	0
Polymeric-immunoglobulin receptor	4	0	0	0	0

THE HUMAN GENOME

alytic activity, as a uracil DNA glycosylase (140) and functions as a cell cycle regulator (141) and has even been implicated in apoptosis (142).

Translation. Another striking set of human expansions has occurred in certain families involved in the translational machinery. We identified 28 different ribosomal subunits that each have at least 10 copies in the genome; on average, for all ribosomal proteins there is about an 8- to 10-fold expansion in the number of genes relative to either the worm or fly. Retrotransposed pseudogenes

may account for many of these expansions [see the discussion above and (143)]. Recent evidence suggests that a number of ribosomal proteins have secondary functions independent of their involvement in protein biosynthesis; for example, L13a and the related L7 subunits (36 copies in humans) have been shown to induce apoptosis (144).

There is also a four- to fivefold expansion in the elongation factor 1- α family (eEF1A; 56 human genes). Many of these expansions likely represent intronless paralogs that have presumably arisen from retro-

transposition, and again there is evidence that many of these may be pseudogenes (145). However, a second form (eEF1A2) of this factor has been identified with tissue-specific expression in skeletal muscle and a complementary expression pattern to the ubiquitously expressed eEF1A (146).

Ribonucleoproteins. Alternative splicing results in multiple transcripts from a single gene, and can therefore generate additional diversity in an organism's protein complement. We have identified 269 genes for ribonucleoproteins. This represents over 2.5 times the number of ribonucleoprotein genes in the worm, two times that of the fly, and about the same as the 265 identified in the *Arabidopsis* genome. Whether the diversity of ribonucleoprotein genes in humans contributes to gene regulation at either the splicing or translational level is unknown.

Posttranslational modifications. In this set of processes, the most prominent expansion is the transglutaminases, calcium-dependent enzymes that catalyze the cross-linking of proteins in cellular processes such as hemostasis and apoptosis (147). The vitamin K-dependent gamma carboxylase gene product acts on the GLA domain (missing in the fly and worm) found in coagulation factors, osteocalcin, and matrix GLA protein (148). Tyrosylprotein sulfotransferases participate in the posttranslational modification of proteins involved in inflammation and hemostasis, including coagulation factors and chemokine receptors (149). Although there is no significant numerical increase in the counts for domains involved in nuclear protein modification, there are a number of domain arrangements in the predicted human proteins that are not found in the other currently sequenced genomes. These include the tandem association of two histone deacetylase domains in HD6 with a ubiquitin finger domain, a feature lacking in the fly genome. An additional example is the co-occurrence of important nuclear regulatory enzyme PARP (poly-ADP ribosyl transferase) domain fused to protein-interaction domains—BRCT and VWA in humans.

Concluding remarks. There are several possible explanations for the differences in phenotypic complexity observed in humans when compared to the fly and worm. Some of these relate to the prominent differences in the immune system, hemostasis, neuronal, vascular, and cytoskeletal complexity. The finding that the human genome contains fewer genes than previously predicted might be compensated for by combinatorial diversity generated at the levels of protein architecture, transcriptional and translational control, posttranslational modification of proteins, or posttranscriptional regulation. Extensive domain shuffling to increase or alter combinatorial diversity can provide an exponential

Table 19 (Continued)

Panther family/subfamily*	H	F	W	Y	A
MHC class I	22	0	0	0	0
MHC class II	20	0	0	0	0
Other immunoglobulin†	114	0	0	0	0
Toll receptor-related	10	6	0	0	0
<i>Developmental and homeostatic regulators</i>					
Signaling molecules†					
Calcitonin	3	0	0	0	0
Ephrin	8	2	4	0	0
FGF	24	1	1	0	0
Glucagon	4	0	0	0	0
Glycoprotein hormone beta chain	2	0	0	0	0
Insulin	1	0	0	0	0
Insulin-like hormone	3	0	0	0	0
Nerve growth factor	3	0	0	0	0
Neuregulin/herregulin	6	0	0	0	0
neuropeptide Y	4	0	0	0	0
PDGF	1	1	0	0	0
Relaxin	3	0	0	0	0
Stannocalcin	2	0	0	0	0
Thymopoietin	2	0	1	0	0
Thymosin beta	4	2	0	0	0
TGF- β	29	6	4	0	0
VEGF	4	0	0	0	0
Wnt	18	6	5	0	0
Receptors†					
Ephrin receptor	12	2	1	0	0
FGF receptor	4	4	0	0	0
Frizzled receptor	12	6	5	0	0
Parathyroid hormone receptor	2	0	0	0	0
VEGF receptor	5	0	0	0	0
BDNF/NT-3 nerve growth factor receptor	4	0	0	0	0
<i>Kinases and phosphatases</i>					
Dual-specificity protein phosphatase	29	8	10	4	11
S/T and dual-specificity protein kinase†	395	198	315	114	1102
S/T protein phosphatase	15	19	51	13	29
Y protein kinase†	106	47	100	5	16
Y protein phosphatase	56	22	95	5	6
<i>Signal transduction</i>					
ARF family	55	29	27	12	45
Cyclic nucleotide phosphodiesterase	25	8	6	1	0
G protein-coupled receptors††	616	146	284	0	1
G-protein alpha	27	10	22	2	5
G-protein beta	5	3	2	1	1
G-protein gamma	13	2	2	0	0
Ras superfamily	141	64	62	26	86
G-protein modulators†					
ARF GTPase-activating	20	8	9	5	15
Neurofibromin	7	2	0	2	0
Ras GTPase-activating	9	3	8	1	0
Tuberlin	7	3	2	0	0
Vav proto-oncogene family	35	15	13	3	0

Table 19 (Continued)

Panther family/subfamily*	H	F	W	Y	A
<i>Transcription factors/chromatin organization</i>					
C2H2 zinc finger-containing†	607	232	79	28	8
COE	7	1	1	0	0
CREB	7	1	2	0	0
ETS-related	25	8	10	0	0
Forkhead-related	34	19	15	4	0
FOS	8	2	1	0	0
Groucho	13	2	1	0	0
Histone H1	5	0	1	0	0
Histone H2A	24	1	17	3	13
Histone H2B	21	1	17	2	12
Histone H3	28	2	24	2	16
Histone H4	9	1	16	1	8
Homeotic†	168	104	74	4	78
ABD-B	5	0	0	0	0
Bithoraxoid	1	8	1	0	0
Iroquois class	7	3	1	0	0
Distal-less	5	2	1	0	0
Engrailed	2	2	1	0	0
LIM-containing	17	8	3	0	0
MEIS/KNOX class	9	4	4	2	26
NK-3/NK-2 class	9	4	5	0	0
Paired box	38	28	23	0	2
Six	5	3	4	0	0
Leucine zipper	6	0	0	0	0
Nuclear hormone receptor†	59	25	183	1	4
Pou-related	15	5	4	1	0
Runt-related	3	4	2	0	0
<i>ECM adhesion</i>					
Cadherin	113	17	16	0	0
Claudin	20	0	0	0	0
Complement receptor-related	22	8	6	0	0
Connexin	14	0	0	0	0
Galectin	12	5	22	0	0
Glypican	13	2	1	0	0
ICAM	6	0	0	0	0
Integrin alpha	24	7	4	0	1
Integrin beta	9	2	2	0	0
LDL receptor family	26	19	20	0	2
Proteoglycans	22	9	7	0	5
<i>Apoptosis</i>					
Bcl-2	12	1	0	0	0
Calpain	22	4	11	1	3
Calpain inhibitor	4	0	0	0	1
Caspase	13	7	3	0	0
<i>Hemostasis</i>					
ADAM/ADAMTS	51	9	12	0	0
Fibronectin	3	0	0	0	0
Globin	10	2	3	0	3
Matrix metalloprotease	19	2	7	0	3
Serum amyloid A	4	0	0	0	0
Serum amyloid P (subfamily of Pentaxin)	2	0	0	0	0
Serum paraoxonase/arylesterase	4	0	3	0	0
Serum albumin	4	0	0	0	0
Transglutaminase	10	1	0	0	0
<i>Other enzymes</i>					
Cytochrome p450	60	89	83	3	256
GAPDH	46	3	4	3	8
Heparan sulfotransferase	11	4	2	0	0
<i>Splicing and translation</i>					
EF-1alpha	56	13	10	6	13
Ribonucleoproteins†	269	135	104	60	265
Ribosomal proteins†	812	111	80	117	256

*The table lists Panther families or subfamilies relevant to the text that either (i) are not specifically represented by Pfam (Table 18) or (ii) differ in counts from the corresponding Pfam models. †This class represents a number of different families in the same Panther molecular function subcategory. ‡This count includes only rhodopsin-class, secretin-class, and metabotropic glutamate-class GPCRs.

increase in the ability to mediate protein-protein interactions without dramatically increasing the absolute size of the protein complement (150). Evolution of apparently new (from the perspective of sequence analysis) protein domains and increasing regulatory complexity by domain accretion both quantitatively and qualitatively (recruitment of novel domains with preexisting ones) are two features that we observe in humans. Perhaps the best illustration of this trend is the C2H2 zinc finger-containing transcription factors, where we see expansion in the number of domains per protein, together with vertebrate-specific domains such as KRAB and SCAN. Recent reports on the prominent use of internal ribosomal entry sites in the human genome to regulate translation of specific classes of proteins suggests that this is an area that needs further research to identify the full extent of this process in the human genome (151). At the posttranslational level, although we provide examples of expansions of some protein families involved in these modifications, further experimental evidence is required to evaluate whether this is correlated with increased complexity in protein processing. Posttranscriptional processing and the extent of isoform generation in the human remain to be cataloged in their entirety. Given the conserved nature of the spliceosomal machinery, further analysis will be required to dissect regulation at this level.

8 Conclusions

8.1 The whole-genome sequencing approach versus BAC by BAC

Experience in applying the whole-genome shotgun sequencing approach to a diverse group of organisms with a wide range of genome sizes and repeat content allows us to assess its strengths and weaknesses. With the success of the method for a large number of microbial genomes, *Drosophila*, and now the human, there can be no doubt concerning the utility of this method. The large number of microbial genomes that have been sequenced by this method (15, 80, 152) demonstrate that megabase-sized genomes can be sequenced efficiently without any input other than the de novo mate-paired sequences. With more complex genomes like those of *Drosophila* or human, map information, in the form of well-ordered markers, has been critical for long-range ordering of scaffolds. For joining scaffolds into chromosomes, the quality of the map (in terms of the order of the markers) is more important than the number of markers per se. Although this mapping could have been performed concurrently with sequencing, the prior existence of mapping data was beneficial. During the sequencing of the *A. thaliana* genome, sequencing of individual BAC clones permitted extension of the se-

quence well into centromeric regions and allowed high-quality resolution of complex repeat regions. Likewise, in *Drosophila*, the BAC physical map was most useful in regions near the highly repetitive centromeres and telomeres. WGA has been found to deliver excellent-quality reconstructions of the unique regions of the genome. As the genome size, and more importantly the repetitive content, increases, the WGA approach delivers less of the repetitive sequence.

The cost and overall efficiency of clone-by-clone approaches makes them difficult to justify as a stand-alone strategy for future large-scale genome-sequencing projects. Specific applications of BAC-based or other clone mapping and sequencing strategies to resolve ambiguities in sequence assembly that cannot be efficiently resolved with computational approaches alone are clearly worth exploring. Hybrid approaches to whole-genome sequencing will only work if there is sufficient coverage in both the whole-genome shotgun phase and the BAC clone sequencing phase. Our experience with human genome assembly suggests that this will require at least 3× coverage of both whole-genome and BAC shotgun sequence data.

8.2 The low gene number in humans

We have sequenced and assembled ~95% of the euchromatic sequence of *H. sapiens* and used a new automated gene prediction method to produce a preliminary catalog of the human genes. This has provided a major surprise: We have found far fewer genes (26,000 to 38,000) than the earlier molecular predictions (50,000 to over 140,000). Whatever the reasons for this current disparity, only detailed annotation, comparative genomics (particularly using the *Mus musculus* genome), and careful molecular dissection of complex phenotypes will clarify this critical issue of the basic "parts list" of our genome. Certainly, the analysis is still incomplete and considerable refinement will occur in the years to come as the precise structure of each transcription unit is evaluated. A good place to start is to determine why the gene estimates derived from EST data are so discordant with our predictions. It is likely that the following contribute to an inflated gene number derived from ESTs: the variable lengths of 3'- and 5'-untranslated leaders and trailers; the little-understood vagaries of RNA processing that often leave intronic regions in an unspliced condition; the finding that nearly 40% of human genes are alternatively spliced (153); and finally, the unsolved technical problems in EST library construction where contamination from heterogeneous nuclear RNA and genomic DNA are not uncommon. Of course, it is possible that there are genes that remain unpredicted owing to the absence of EST or protein data to support them, although our use of mouse genome data for

predicting genes should limit this number. As was true at the beginning of genome sequencing, ultimately it will be necessary to measure mRNA in specific cell types to demonstrate the presence of a gene.

J. B. S. Haldane speculated in 1937 that a population of organisms might have to pay a price for the number of genes it can possibly carry. He theorized that when the number of genes becomes too large, each zygote carries so many new deleterious mutations that the population simply cannot maintain itself. On the basis of this premise, and on the basis of available mutation rates and x-ray-induced mutations at specific loci, Muller, in 1967 (154), calculated that the mammalian genome would contain a maximum of not much more than 30,000 genes (155). An estimate of 30,000 gene loci for humans was also arrived at by Crow and Kimura (156). Muller's estimate for *D. melanogaster* was 10,000 genes, compared to 13,000 derived by annotation of the fly genome (26, 27). These arguments for the theoretical maximum gene number were based on simplified ideas of genetic load—that all genes have a certain low rate of mutation to a deleterious state. However, it is clear that many mouse, fly, worm, and yeast knockout mutations lead to almost no discernible phenotypic perturbations.

The modest number of human genes means that we must look elsewhere for the mechanisms that generate the complexities inherent in human development and the sophisticated signaling systems that maintain homeostasis. There are a large number of ways in which the functions of individual genes and gene products are regulated. The degree of "openness" of chromatin structure and hence transcriptional activity is regulated by protein complexes that involve histone and DNA enzymatic modifications. We enumerate many of the proteins that are likely involved in nuclear regulation in Table 19. The location, timing, and quantity of transcription are intimately linked to nuclear signal transduction events as well as by the tissue-specific expression of many of these proteins. Equally important are regulatory DNA elements that include insulators, repeats, and endogenous viruses (157); methylation of CpG islands in imprinting (158); and promoter-enhancer and intronic regions that modulate transcription. The spliceosomal machinery consists of multisubunit proteins (Table 19) as well as structural and catalytic RNA elements (159) that regulate transcript structure through alternative start and termination sites and splicing. Hence, there is a need to study different classes of RNA molecules (160) such as small nucleolar RNAs, antisense riboregulator RNA, RNA involved in X-dosage compensation, and other structural RNAs to appreciate their precise role in regulating gene expression. The phenomenon

of RNA editing in which coding changes occur directly at the level of mRNA is of clinical and biological relevance (161). Finally, examples of translational control include internal ribosomal entry sites that are found in proteins involved in cell cycle regulation and apoptosis (162). At the protein level, minor alterations in the nature of protein-protein interactions, protein modifications, and localization can have dramatic effects on cellular physiology (163). This dynamic system therefore has many ways to modulate activity, which suggests that definition of complex systems by analysis of single genes is unlikely to be entirely successful.

In situ studies have shown that the human genome is asymmetrically populated with G+C content, CpG islands, and genes (68). However, the genes are not distributed quite as unequally as had been predicted (Table 9) (69). The most G+C-rich fraction of the genome, H3 isochores, constitute more of the genome than previously thought (about 9%), and are the most gene-dense fraction, but contain only 25% of the genes, rather than the predicted ~40%. The low G+C L isochores make up 65% of the genome, and 48% of the genes. This inhomogeneity, the net result of millions of years of mammalian gene duplication, has been described as the "desertification" of the vertebrate genome (71). Why are there clustered regions of high and low gene density, and are these accidents of history or driven by selection and evolution? If these deserts are dispensable, it ought to be possible to find mammalian genomes that are far smaller in size than the human genome. Indeed, many species of bats have genome sizes that are much smaller than that of humans; for example, *Miniopterus*, a species of Italian bat, has a genome size that is only 50% that of humans (164). Similarly, *Muntiacus*, a species of Asian barking deer, has a genome size that is ~70% that of humans.

8.3 Human DNA sequence variation and its distribution across the genome

This is the first eukaryotic genome in which a nearly uniform ascertainment of polymorphism has been completed. Although we have identified and mapped more than 3 million SNPs, this by no means implies that the task of finding and cataloging SNPs is complete. These represent only a fraction of the SNPs present in the human population as a whole. Nevertheless, this first glimpse at genome-wide variation has revealed strong inhomogeneities in the distribution of SNPs across the genome. Polymorphism in DNA carries with it a snapshot of the past operation of population genetic forces, including mutation, migration, selection, and genetic drift. The availability of a dense array of SNPs will allow questions related to each of these factors to be addressed on a genome-wide basis. SNP studies can establish the range of haplo-

types present in subjects of different ethnogeographic origins, providing insights into population history and migration patterns. Although such studies have suggested that modern human lineages derive from Africa, many important questions regarding human origins remain unanswered, and more analyses using detailed SNP maps will be needed to settle these controversies. In addition to providing evidence for population expansions, migration, and admixture, SNPs can serve as markers for the extent of evolutionary constraint acting on particular genes. The correlation between patterns of intraspecies and interspecies genetic variation may prove to be especially informative to identify sites of reduced genetic diversity that may mark loci where sequence variations are not tolerated.

The remarkable heterogeneity in SNP density implies that there are a variety of forces acting on polymorphism—sparse regions may have lower SNP density because the mutation rate is lower, because most of those regions have a lower fraction of mutations that are tolerated, or because recent strong selection in favor of a newly arisen allele “swept” the linked variation out of the population (165). The effect of random genetic drift also varies widely across the genome. The nonrecombining portion of the Y chromosome faces the strongest pressure from random drift because there are roughly one-quarter as many Y chromosomes in the population as there are autosomal chromosomes, and the level of polymorphism on the Y is correspondingly less. Similarly, the X chromosome has a smaller effective population size than the autosomes, and its nucleotide diversity is also reduced. But even across a single autosome, the effective population size can vary because the density of deleterious mutations may vary. Regions of high density of deleterious mutations will see a greater rate of elimination by selection, and the effective population size will be smaller (166). As a result, the density of even completely neutral SNPs will be lower in such regions. There is a large literature on the association between SNP density and local recombination rates in *Drosophila*, and it remains an important task to assess the strength of this association in the human genome, because of its impact on the design of local SNP densities for disease-association studies. It also remains an important task to validate SNPs on a genomic scale in order to assess the degree of heterogeneity among geographic and ethnic populations.

8.4 Genome complexity

We will soon be in a position to move away from the cataloging of individual components of the system, and beyond the simplistic notions of “this binds to that, which

then docks on this, and then the complex moves there. . .” (167) to the exciting area of network perturbations, nonlinear responses and thresholds, and their pivotal role in human diseases.

The enumeration of other “parts lists” reveals that in organisms with complex nervous systems, neither gene number, neuron number, nor number of cell types correlates in any meaningful manner with even simplistic measures of structural or behavioral complexity. Nor would they be expected to; this is the realm of nonlinearities and epigenesis (168). The 520 million neurons of the common octopus exceed the neuronal number in the brain of a mouse by an order of magnitude. It is apparent from a comparison of genomic data on the mouse and human, and from comparative mammalian neuroanatomy (169), that the morphological and behavioral diversity found in mammals is underpinned by a similar gene repertoire and similar neuroanatomies. For example, when one compares a pygmy marmoset (which is only 4 inches tall and weighs about 6 ounces) to a chimpanzee, the brain volume of this minute primate is found to be only about 1.5 cm³, two orders of magnitude less than that of a chimp and three orders less than that of humans. Yet the neuroanatomies of all three brains are strikingly similar, and the behavioral characteristics of the pygmy marmoset are little different from those of chimpanzees. Between humans and chimpanzees, the gene number, gene structures and functions, chromosomal and genomic organizations, and cell types and neuroanatomies are almost indistinguishable, yet the developmental modifications that predisposed human lineages to cortical expansion and development of the larynx, giving rise to language, culminated in a massive singularity that by even the simplest of criteria made humans more complex in a behavioral sense.

Simple examination of the number of neurons, cell types, or genes or of the genome size does not alone account for the differences in complexity that we observe. Rather, it is the interactions within and among these sets that result in such great variation. In addition, it is possible that there are “special cases” of regulatory gene networks that have a disproportionate effect on the overall system. We have presented several examples of “regulatory genes” that are significantly increased in the human genome compared with the fly and worm. These include extracellular ligands and their cognate receptors (e.g., wnt, frizzled, TGF- β , ephrins, and connexins), as well as nuclear regulators (e.g., the KRAB and homeodomain transcription factor families), where a few proteins control broad developmental processes. The answers to these “complexities” perhaps lie in these expanded gene families and differences in the regulatory control of ancient genes, proteins, pathways, and cells.

8.5 Beyond single components

While few would disagree with the intuitive conclusion that Einstein’s brain was more complex than that of *Drosophila*, closer comparisons such as whether the set of predicted human proteins is more complex than the protein set of *Drosophila*, and if so, to what degree, are not straightforward, since protein, protein domain, or protein-protein interaction measures do not capture context-dependent interactions that underpin the dynamics underlying phenotype.

Currently, there are more than 30 different mathematical descriptions of complexity (170). However, we have yet to understand the mathematical dependency relating the number of genes with organism complexity. One pragmatic approach to the analysis of biological systems, which are composed of nonidentical elements (proteins, protein complexes, interacting cell types, and interacting neuronal populations), is through graph theory (171). The elements of the system can be represented by the vertices of complex topographies, with the edges representing the interactions between them. Examination of large networks reveals that they can self-organize, but more important, they can be particularly robust. This robustness is not due to redundancy, but is a property of inhomogeneously wired networks. The error tolerance of such networks comes with a price; they are vulnerable to the selection or removal of a few nodes that contribute disproportionately to network stability. Gene knockouts provide an illustration. Some knockouts may have minor effects, whereas others have catastrophic effects on the system. In the case of vimentin, a supposedly critical component of the cytoplasmic intermediate filament network of mammals, the knockout of the gene in mice reveals them to be reproductively normal, with no obvious phenotypic effects (172), and yet the usually conspicuous vimentin network is completely absent. On the other hand, ~30% of knockouts in *Drosophila* and mice correspond to critical nodes whose reduction in gene product, or total elimination, causes the network to crash most of the time, although even in some of these cases, phenotypic normalcy ensues, given the appropriate genetic background. Thus, there are no “good” genes or “bad” genes, but only networks that exist at various levels and at different connectivities, and at different states of sensitivity to perturbation. Sophisticated mathematical analysis needs to be constantly evaluated against hard biological data sets that specifically address network dynamics. Nowhere is this more critical than in attempts to come to grips with “complexity,” particularly because deconvoluting and correcting complex networks that have undergone perturbation, and have resulted in human diseases, is the greatest significant challenge now facing us.

It has been predicted for the last 15 years that complete sequencing of the human ge-

nome would open up new strategies for human biological research and would have a major impact on medicine, and through medicine and public health, on society. Effects on biomedical research are already being felt. This assembly of the human genome sequence is but a first, hesitant step on a long and exciting journey toward understanding the role of the genome in human biology. It has been possible only because of innovations in instrumentation and software that have allowed automation of almost every step of the process from DNA preparation to annotation. The next steps are clear: We must define the complexity that ensues when this relatively modest set of about 30,000 genes is expressed. The sequence provides the framework upon which all the genetics, biochemistry, physiology, and ultimately phenotype depend. It provides the boundaries for scientific inquiry. The sequence is only the first level of understanding of the genome. All genes and their control elements must be identified; their functions, in concert as well as in isolation, defined; their sequence variation worldwide described; and the relation between genome variation and specific phenotypic characteristics determined. Now we know what we have to explain.

Another paramount challenge awaits: public discussion of this information and its potential for improvement of personal health. Many diverse sources of data have shown that any two individuals are more than 99.9% identical in sequence, which means that all the glorious differences among individuals in our species that can be attributed to genes falls in a mere 0.1% of the sequence. There are two fallacies to be avoided: determinism, the idea that all characteristics of the person are "hard-wired" by the genome; and reductionism, the view that with complete knowledge of the human genome sequence, it is only a matter of time before our understanding of gene functions and interactions will provide a complete causal description of human variability. The real challenge of human biology, beyond the task of finding out how genes orchestrate the construction and maintenance of the miraculous mechanism of our bodies, will lie ahead as we seek to explain how our minds have come to organize thoughts sufficiently well to investigate our own existence.

References and Notes

1. R. L. Sinsheimer, *Genomics* 5, 954 (1989); U.S. Department of Energy, Office of Health and Environmental Research, *Sequencing the Human Genome: Summary Report of the Santa Fe Workshop*, Santa Fe, NM, 3 to 4 March 1986 (Los Alamos National Laboratory, Los Alamos, NM, 1986).
2. R. Cook-Deegan, *The Gene Wars: Science, Politics, and the Human Genome* (Norton, New York, 1996).
3. F. Sanger et al., *Nature* 265, 687 (1977).
4. P. H. Seeburg et al., *Trans. Assoc. Am. Physicians* 90, 109 (1977).

5. E. C. Strauss, J. A. Kobori, G. Siu, L. E. Hood, *Anal. Biochem.* 154, 353 (1986).
6. J. Gocayne et al., *Proc. Natl. Acad. Sci. U.S.A.* 84, 8296 (1987).
7. A. Martin-Gallardo et al., *DNA Sequence* 3, 237 (1992); W. R. McCombie et al., *Nature Genet.* 1, 348 (1992); M. A. Jensen et al., *DNA Sequence* 1, 233 (1991).
8. M. D. Adams et al., *Science* 252, 1651 (1991).
9. M. D. Adams et al., *Nature* 355, 632 (1992); M. D. Adams, A. R. Kerlavage, C. Fields, J. C. Venter, *Nature Genet.* 4, 256 (1993); M. D. Adams, M. B. Soares, A. R. Kerlavage, C. Fields, J. C. Venter, *Nature Genet.* 4, 373 (1993); M. H. Polymeropoulos et al., *Nature Genet.* 4, 381 (1993); M. Marra et al., *Nature Genet.* 21, 191 (1999).
10. M. D. Adams et al., *Nature* 377, 3 (1995); O. White et al., *Nucleic Acids Res.* 21, 3829 (1993).
11. F. Sanger, A. R. Coulson, G. F. Hong, D. F. Hill, G. B. Petersen, *J. Mol. Biol.* 162, 729 (1982).
12. B. W. J. Mahy, J. J. Esposito, J. C. Venter, *Am. Soc. Microbiol. News* 57, 577 (1991).
13. R. D. Fleischmann et al., *Science* 269, 496 (1995).
14. C. M. Fraser et al., *Science* 270, 397 (1995).
15. C. J. Bult et al., *Science* 273, 1058 (1996); J. F. Tomb et al., *Nature* 388, 539 (1997); H. P. Klenk et al., *Nature* 390, 364 (1997).
16. J. C. Venter, H. O. Smith, L. Hood, *Nature* 381, 364 (1996).
17. H. Schmitt et al., *Genomics* 33, 9 (1996).
18. S. Zhao et al., *Genomics* 63, 321 (2000).
19. X. Lin et al., *Nature* 402, 761 (1999).
20. J. L. Weber, E. W. Myers, *Genome Res.* 7, 401 (1997).
21. P. Green, *Genome Res.* 7, 410 (1997).
22. E. Pennisi, *Science* 280, 1185 (1998).
23. J. C. Venter et al., *Science* 280, 1540 (1998).
24. M. D. Adams et al., *Nature* 368, 474 (1994).
25. E. Marshall, E. Pennisi, *Science* 280, 994 (1998).
26. M. D. Adams et al., *Science* 287, 2185 (2000).
27. G. M. Rubin et al., *Science* 287, 2204 (2000).
28. E. W. Myers et al., *Science* 287, 2196 (2000).
29. F. S. Collins et al., *Science* 282, 682 (1998).
30. International Human Genome Sequencing Consortium (2001), *Nature* 409, 860 (2001).
31. Institutional review board: P. Calabresi (chairman), H. P. Freeman, C. McCarthy, A. L. Caplan, G. D. Rogell, J. Karp, M. K. Evans, B. Margus, C. L. Carter, R. A. Millman, S. Broder.
32. Eligibility criteria for participation in the study were as follows: prospective donors had to be 21 years of age or older, not pregnant, and capable of giving an informed consent. Donors were asked to self-define their ethnic backgrounds. Standard blood bank screens (screening for HIV, hepatitis viruses, and so forth) were performed on all samples at the clinical laboratory prior to DNA extraction in the Celera laboratory. All samples that tested positive for transmissible viruses were ineligible and were discarded. Karyotype analysis was performed on peripheral blood lymphocytes from all samples selected for sequencing; all were normal. A two-staged consent process for prospective donors was employed. The first stage of the consent process provided information about the genome project, procedures, and risks and benefits of participating. The second stage of the consent process involved answering follow-up questions and signing consent forms, and was conducted about 48 hours after the first.
33. DNA was isolated from blood (173) or sperm. For sperm, a washed pellet (100 μ l) was lysed in a suspension (1 ml) containing 0.1 M NaCl, 10 mM tris-Cl-20 mM EDTA (pH 8), 1% SDS, 1 mg proteinase K, and 10 mM dithiothreitol for 1 hour at 37°C. The lysate was extracted with aqueous phenol and with phenol/chloroform. The DNA was ethanol precipitated and dissolved in 1 ml TE buffer. To make genomic libraries, DNA was randomly sheared, end-polished with consecutive BAL31 nuclease and T4 DNA polymerase treatments, and size-selected by electrophoresis on 1% low-melting-point agarose. After ligation to Bst XI adapters (Invitrogen, catalog no. N408-18), DNA was purified by three rounds of gel electrophoresis to remove excess adapters, and the fragments, now with 3'-CACA overhangs, were inserted into Bst XI-linearized plasmid vector with 3'-TGTG overhangs. Libraries with three different average sizes of inserts were constructed: 2, 10, and 50 kbp. The 2-kbp fragments were cloned in a high-copy pUC18 derivative. The 10- and 50-kbp fragments were cloned in a medium-copy pBR322 derivative. The 2- and 10-kbp libraries yielded uniform-sized large colonies on plating. However, the 50-kbp libraries produced many small colonies and inserts were unstable. To remedy this, the 50-kbp libraries were digested with Bgl II, which does not cleave the vector, but generally cleaved several times within the 50-kbp insert. A 1264-bp Bam-HI kanamycin resistance cassette (purified from pUCK4; Amersham Pharmacia, catalog no. 27-4958-01) was added and ligation was carried out at 37°C in the continual presence of Bgl II. As Bgl II-Bgl II ligations occurred, they were continually cleaved, whereas Bam-HI-Bgl II ligations were not cleaved. A high yield of internally deleted circular library molecules was obtained in which the residual insert ends were separated by the kanamycin cassette DNA. The internally deleted libraries, when plated on agar containing ampicillin (50 μ g/ml), carbenicillin (50 μ g/ml), and kanamycin (15 μ g/ml), produced relatively uniform large colonies. The resulting clones could be prepared for sequencing using the same procedures as clones from the 10-kbp libraries.
34. Transformed cells were plated on agar diffusion plates prepared with a fresh top layer containing no antibiotic poured on top of a previously set bottom layer containing excess antibiotic, to achieve the correct final concentration. This method of plating permitted the cells to develop antibiotic resistance before being exposed to antibiotic without the potential clone bias that can be introduced through liquid outgrowth protocols. After colonies had grown, QBot (Genetix, UK) automated colony-picking robots were used to pick colonies meeting stringent size and shape criteria and to inoculate 384-well microtiter plates containing liquid growth medium. Liquid cultures were incubated overnight, with shaking, and were scored for growth before passing to template preparation. Template DNA was extracted from liquid bacterial culture using a procedure based upon the alkaline lysis miniprep method (173) adapted for high throughput processing in 384-well microtiter plates. Bacterial cells were lysed; cell debris was removed by centrifugation; and plasmid DNA was recovered by isopropanol precipitation and resuspended in 10 mM tris-HCl buffer. Reagent dispensing operations were accomplished using Titertek MAP 8 liquid dispensing systems. Plate-to-plate liquid transfers were performed using Tomtec Quadra 384 Model 320 pipetting robots. All plates were tracked throughout processing by unique plate barcodes. Mated sequencing reads from opposite ends of each clone insert were obtained by preparing two 384-well cycle sequencing reaction plates from each plate of plasmid template DNA using ABI-PRISM BigDye Terminator chemistry (Applied Biosystems) and standard M13 forward and reverse primers. Sequencing reactions were prepared using the Tomtec Quadra 384-320 pipetting robot. Parent-child plate relationships and, by extension, forward-reverse sequence mate pairs were established by automated plate barcode reading by the onboard barcode reader and were recorded by direct LIMS communication. Sequencing reaction products were purified by alcohol precipitation and were dried, sealed, and stored at 4°C in the dark until needed for sequencing, at which time the reaction products were resuspended in deionized formamide and sealed immediately to prevent degradation. All sequence data were generated using a single sequencing platform, the ABI PRISM 3700 DNA Analyzer. Sample sheets were created at load time using a Java-based application that facilitates barcode scanning of the sequencing plate barcode, retrieves sample information from the central LIMS, and reserves unique trace identifiers. The application permitted a single sample sheet file in the linking directory and deleted previously created sample sheet files immediately upon scanning of a

- sample plate barcode, thus enhancing sample sheet-to-plate associations.
35. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977); J. M. Prober et al., *Science* **238**, 336 (1987).
 36. Celera's computing environment is based on Compaq Computer Corporation's Alpha system technology running the Tru64 Unix operating system. Celera uses these Alphas as Data Servers and as nodes in a Virtual Compute Farm, all of which are connected to a fully switched network operating at Fast Ethernet speed (for the VCF) and gigabit Ethernet speed (for data servers). Load balancing and scheduling software manages the submission and execution of jobs, based on central processing unit (CPU) speed, memory requirements, and priority. The Virtual Compute Farm is composed of 440 Alpha CPUs, which includes model EV6 running at a clock speed of 400 MHz and EV67 running at 667 MHz. Available memory on these systems ranges from 2 GB to 8 GB. The VCF is used to manage trace file processing, and annotation. Genome assembly was performed on a GS 160 running 16 EV67s (667 MHz) and 64 GB of memory, and 10 ES40s running 4 EV6s (500 MHz) and 32 GB of memory. A total of 100 terabytes of physical disk storage was included in a Storage Area Network that was available to systems across the environment. To ensure high availability, file and database servers were configured as 4-node Alpha TruClusters, so that services would fail over in the event of hardware or software failure. Data availability was further enhanced by using hardware- and software-based disk mirroring (RAID-0), disk striping (RAID-1), and disk striping with parity (RAID-5).
 37. Trace processing generates quality values for base calls by means of Paracel's TraceTuner, trims sequence reads according to quality values, trims vector and adapter sequence from high-quality reads, and screens sequences for contaminants. Similar in design and algorithm to the phred program (174), TraceTuner reports quality values that reflect the log-odds score of each base being correct. Read quality was evaluated in 50-bp windows, each read being trimmed to include only those consecutive 50-bp segments with a minimum mean accuracy of 97%. End windows (both ends of the trace) of 1, 5, 10, 25, and 50 bases were trimmed to a minimum mean accuracy of 98%. Every read was further checked for vector and contaminant matches of 50 bp or more, and if found, the read was removed from consideration. Finally, any match to the 5' vector splice junction in the initial part of a read was removed.
 38. National Center for Biotechnology Information (NCBI); available at www.ncbi.nlm.nih.gov/.
 39. NCBI; available at www.ncbi.nlm.nih.gov/HTGS/.
 40. All baccigs over 3 kbp were examined for coverage by Celera mate pairs. An interval of a baccig was deemed an assembly error where there were no mate pairs spanning the interval and at least two reads that should have their mate on the other side of the interval but did not. In other words, there was no mate pair evidence supporting a join in the breakpoint interval and at least two mate pairs contradicting the join. By this criterion, we detected and broke apart baccigs at 13,037 locations, or equivalently, we found 2.13% of the baccigs to be misassembled.
 41. We considered a BAC entry to be chimeric if, by the Lander-Waterman statistic (175), the odds were 0.99 or more that the assembly we produced was inconsistent with the sequence coming from a single source. By this criterion, 714 or 2.2% of BAC entries were deemed chimeric.
 42. G. Myers, S. Selznick, Z. Zhang, W. Miller, J. Comput. Biol. **3**, 563 (1996).
 43. E. W. Myers, J. L. Weber, in *Computational Methods in Genome Research*, S. Suhai, Ed. (Plenum, New York, 1996), pp. 73-89.
 44. P. Deloukas et al., *Science* **282**, 744 (1998).
 45. M. A. Marra et al., *Genome Res.* **7**, 1072 (1997).
 46. J. Zhang et al., data not shown.
 47. Shredded baccigs were located on long CSA scaffolds (>500 kbp) and the distribution of these fragments on the scaffolds was analyzed. If the spread of these fragments was greater than four times the reported BAC length, the BAC was considered to be chimeric. In addition, if >20% of baccigs of a given BAC were found on a different scaffolds that were not adjacent in map position, then the BAC was also considered as chimeric. The total chimeric BACs divided by the number of BACs used for CSA gave the minimal estimate of chimerism rate.
 48. M. Hattori et al., *Nature* **405**, 311 (2000).
 49. I. Dunham et al., *Nature* **402**, 489 (1999).
 50. A. B. Carvalho, B. P. Lazzaro, A. G. Clark, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13239 (2000).
 51. The International RH Mapping Consortium, available at www.ncbi.nlm.nih.gov/genemap99/.
 52. See <http://ftp.genome.washington.edu/RM/RepeatMasker.html>.
 53. G. D. Schuler, *Trends Biotechnol.* **16**, 456 (1998).
 54. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990).
 - 55a. M. Olivier et al., *Science* **291**, 1298 (2001).
 - 55b. See <http://genome.ucsc.edu/>.
 56. N. Chaudhuri, W. E. Hahn, *Science* **220**, 924 (1983); R. J. Milner, J. G. Sutcliffe, *Nucleic Acids Res.* **11**, 5497 (1983).
 57. D. Dickson, *Nature* **401**, 311 (1999).
 58. B. Ewing, P. Green, *Nature Genet.* **25**, 232 (2000).
 59. H. Roest Crolius et al., *Nature Genet.* **25**, 235 (2000).
 60. M. Yandell, in preparation.
 61. K. D. Pruitt, K. S. Katz, H. Sicotte, D. R. Maglott, *Trends Genet.* **16**, 44 (2000).
 62. Scaffolds containing greater than 10 kbp of sequence were analyzed for features of biological importance through a series of computational steps, and the results were stored in a relational database. For scaffolds greater than one megabase, the sequence was cut into single megabase pieces before computational analysis. All sequence was masked for complex repeats using RepeatMasker (52) before gene finding or homology-based analysis. The computational pipeline required ~7 hours of CPU time per megabase, including repeat masking, or a total compute time of about 20,000 CPU hours. Protein searches were performed against the nonredundant protein database available at the NCBI. Nucleotide searches were performed against human, mouse, and rat Celera Gene Indices (assemblies of cDNA and EST sequences), mouse genomic DNA reads generated at Celera (3X), the Ensembl gene database available at the European Bioinformatics Institute (EBI), human and rodent (mouse and rat) EST data sets parsed from the dbEST database (NCBI), and a curated subset of the RefSeq experimental mRNA database (NCBI). Initial searches were performed on repeat-masked sequence with BLAST 2.0 (54) optimized for the Compaq Alpha computer server and an effective database size of 3×10^9 for BLASTN searches and 1×10^9 for BLASTX searches. Additional processing of each query-subject pair was performed to improve the alignments. All protein BLAST results having an expectation score of $<1 \times 10^{-4}$, human nucleotide BLAST results having an expectation score of $<1 \times 10^{-8}$ with >94% identity, and rodent nucleotide BLAST results having an expectation score of $<1 \times 10^9$ with >80% identity were then examined on the basis of their high-scoring pair (HSP) coordinates on the scaffold to remove redundant hits, retaining hits that supported possible alternative splicing. For BLASTX searches, analysis was performed separately for selected model organisms (yeast, mouse, human, *C. elegans*, and *D. melanogaster*) so as not to exclude HSPs from these organisms that support the same gene structure. Sequences producing BLAST hits judged to be informative, nonredundant, and sufficiently similar to the scaffold sequence were then realigned to the genomic sequence with Sim4 for ESTs, and with Lap for proteins. Because both of these algorithms take splicing into account, the resulting alignments usually give a better representation of intron-exon boundaries than standard BLAST analyses and thus facilitate further annotation (both machine and human). In addition to the homology-based analysis described above, three ab initio gene prediction programs were used (63).
 63. E. C. Uberbacher, Y. Xu, R. J. Mural, *Methods Enzymol.* **266**, 259 (1996); C. Burge, S. Karlin, *J. Mol. Biol.* **268**, 78 (1997); R. J. Mural, *Methods Enzymol.* **303**, 77 (1999); A. A. Salamov, V. V. Solovyev, *Genome Res.* **10**, 516 (2000); Floreal et al., *Genome Res.* **8**, 967 (1998).
 64. G. L. Miklos, B. John, *Am. J. Hum. Genet.* **31**, 264 (1979); U. Francke, *Cytogenet. Cell Genet.* **65**, 206 (1994).
 65. P. E. Warburton, H. F. Willard, in *Human Genome Evolution*, M. S. Jackson, T. Strachan, G. Dover, Eds. (BIOS Scientific, Oxford, 1996), pp. 121-145.
 66. J. E. Horvath, S. Schwartz, E. E. Eichler, *Genome Res.* **10**, 839 (2000).
 67. W. A. Bickmore, A. T. Sumner, *Trends Genet.* **5**, 144 (1989).
 68. G. P. Holmquist, *Am. J. Hum. Genet.* **51**, 17 (1992).
 69. G. Bernardi, *Gene* **241**, 3 (2000).
 70. S. Zoubak, O. Clay, G. Bernardi, *Gene* **174**, 95 (1996).
 71. S. Ohno, *Trends Genet.* **1**, 160 (1985).
 72. K. W. Broman, J. C. Murray, V. C. Sheffield, R. L. White, J. L. Weber, *Am. J. Hum. Genet.* **63**, 861 (1998).
 73. M. J. McEachern, A. Krauskopf, E. H. Blackburn, *Annu. Rev. Genet.* **34**, 331 (2000).
 74. A. Bird, *Trends Genet.* **3**, 342 (1987).
 75. M. Gardiner-Garden, M. Frommer, *J. Mol. Biol.* **196**, 261 (1987).
 76. F. Larsen, G. Gundersen, R. Lopez, H. Prydz, *Genomics* **13**, 1095 (1992).
 77. S. H. Cross, A. Bird, *Curr. Opin. Genet. Dev.* **5**, 309 (1995).
 78. J. Peters, *Genome Biol.* **1**, reviews1028.1 (2000) (<http://genomebiology.com/2000/1/5/reviews/1028>).
 79. C. Grunau, W. Hindermann, A. Rosenthal, *Hum. Mol. Genet.* **9**, 2651 (2000).
 80. F. Antequera, A. Bird, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11995 (1993).
 81. S. H. Cross et al., *Mamm. Genome* **11**, 373 (2000).
 82. D. Slavov et al., *Gene* **247**, 215 (2000).
 83. A. F. Smit, A. D. Riggs, *Nucleic Acids Res.* **23**, 98 (1995).
 84. D. J. Elliott et al., *Hum. Mol. Genet.* **9**, 2117 (2000).
 85. A. V. Makeyev, A. N. Chkheidze, S. A. Liehaber, *J. Biol. Chem.* **274**, 24849 (1999).
 86. Y. Pan, W. K. Decker, A. H. H. M. Huq, W. J. Craig, *Genomics* **59**, 282 (1999).
 87. P. Nouvel, *Genetica* **93**, 191 (1994).
 88. I. Goncalves, L. Duret, D. Mouchiroud, *Genome Res.* **10**, 672 (2000).
 89. Lek first compares all proteins in the proteome to one another. Next, the resulting BLAST reports are parsed, and a graph is created wherein each protein constitutes a node; any hit between two proteins with an expectation beneath a user-specified threshold constitutes an edge. Lek then uses this graph to compute a similarity between each protein pair *ij* in the context of the graph as a whole by simply dividing the number of BLAST hits shared in common between the two proteins by the total number of proteins hit by *i* and *j*. This simple metric has several interesting properties. First, because the similarity metric takes into account both the similarity and the differences between the two sequences at the level of BLAST hits, the metric respects the multidomain nature of protein space. Two multidomain proteins, for instance, each containing domains A and B, will have a greater pairwise similarity to each other than either one will have to a protein containing only A or B domains, so long as A-B-containing multidomain proteins are less frequent in the proteome than are single-domain proteins containing A or B domains. A second interesting property of this similarity metric is that it can be used to produce a similarity matrix for the proteome as a whole without having to first produce a multiple alignment for each protein family, an error-prone and very time-consuming process. Finally, the metric does not require that either sequence have significant homology to the other in order to have a defined similarity to each other, only that they

share at least one significant BLAST hit in common. This is an especially interesting property of the metric, because it allows the rapid recovery of protein families from the proteome for which no multiple alignment is possible, thus providing a computational basis for the extension of protein homology searches beyond those of current HMM- and profile-based search methods. Once the whole-proteome similarity matrix has been calculated, Lek first partitions the proteome into single-linkage clusters (27) on the basis of one or more shared BLAST hits between two sequences. Next, these single-linkage clusters are further partitioned into subclusters, each member of which shares a user-specified pairwise similarity with the other members of the cluster, as described above. For the purposes of this publication, we have focused on the analysis of single-linkage clusters and what we have termed "complete clusters," e.g., those subclusters for which every member has a similarity metric of 1 to every other member of the subcluster. We believe that the single-linkage and complete clusters are of special interest, in part, because they allow us to estimate and to compare sizes of core protein sets in a rigorous manner. The rationale for this is as follows: if one imagines for a moment a perfect clustering algorithm capable of perfectly partitioning one or more perfectly annotated protein sets into protein families, it is reasonable to assume that the number of clusters will always be greater than, or equal to, the number of single-linkage clusters, because single-linkage clustering is a maximally agglomerative clustering method. Thus, if there exists a single protein in the predicted protein set containing domains A and B, then it will be clustered by single linkage together with all single-domain proteins containing domains A or B. Likewise, for a predicted protein set containing a single multidomain protein, the number of real clusters must always be less than or equal to the number of complete clusters, because it is impossible to place a unique multidomain protein into a complete cluster. Thus, the single-linkage and complete clusters plus singletons should comprise a lower and upper bound of sizes of core protein sets, respectively, allowing us to compare the relative size and complexity of different organisms' predicted protein set.

90. T. F. Smith, M. S. Waterman, *J. Mol. Biol.* 147, 195 (1981).
91. A. L. Delcher et al., *Nucleic Acids Res.* 27, 2369 (1999).
92. *Arabidopsis* Genome Initiative, *Nature* 408, 796 (2000).
93. The probability that a contiguous set of proteins is the result of a segmental duplication can be estimated approximately as follows. Given that protein A and B occur on one chromosome, and that A' and B' (paralogs of A and B) also exist in the genome, the probability that B' occurs immediately after A' is $1/N$, where N is the number of proteins in the set (for this analysis, $N = 26,588$). Allowing for B' to occur as any of the next $J-1$ proteins [leaving a gap between A' and B' increases the probability to $(J-1)/N$; allowing B'A' or A'B' gives a probability of $2(J-1)/N$]. Considering three genes ABC, the probability of observing A'B'C' elsewhere in the genome, given that the paralogs exist, is $1/N^2$. Three proteins can occur across a spread of five positions in six ways; more generally, we compute the number of ways that K proteins can be spread across J positions by counting all possible arrangements of $K-2$ proteins in the $J-2$ positions between the first and last protein. Allowing for a spread to vary from K positions (no gaps) to J gives

$$L = \sum_{x=K-2}^{J-2} \binom{J-x}{K-x}$$

arrangements. Thus, the probability of chance occurrence is L/N^{K-1} . Allowing for both sets of genes (e.g., ABC and A'B'C') to be spread across J positions increases this to L^2/N^{K-1} . The duplicated segment might be rearranged by the operations of reversal or translocation; allowing for M such rearrangements gives us a probability $P = L^2 M/N^{K-1}$. For example, the

probability of observing a duplicated set of three genes in two different locations, where the three genes occur across a spread of five positions in both locations, is $36/N^2$; the expected number of such matched sets in the predicted protein set is approximately $(N)(36/N^2) = 36/N$, a value $\ll 1$. Therefore, any such duplications of three genes are unlikely to result from random rearrangements of the genome. If any of the genes occur in more than two copies, the probability that the apparent duplication has occurred by chance increases. The algorithm for selecting candidate duplications only generates matched protein sets with $P \ll 1$.

94. B. J. Trask et al., *Hum. Mol. Genet.* 7, 13 (1998); D. Sharon et al., *Genomics* 61, 24 (1999).
95. W. B. Barbazuk et al., *Genome Res.* 10, 1351 (2000); A. McLysaght, A. J. Enright, L. Skrabaneck, K. H. Wolfe, *Yeast* 17, 22 (2000); D. W. Burt et al., *Nature* 402, 411 (1999).
96. Reviewed in L. Skrabaneck, K. H. Wolfe, *Curr. Opin. Genet. Dev.* 8, 694 (1998).
97. P. Taillon-Miller, Z. Gu, Q. Li, L. Hillier, P. Y. Kwok, *Genome Res.* 8, 748 (1998); P. Taillon-Miller, E. E. Piemot, P. Y. Kwok, *Genome Res.* 9, 499 (1999).
98. D. Altshuler et al., *Nature* 407, 513 (2000).
99. G. T. Marth et al., *Nature Genet.* 23, 452 (1999).
100. W.-H. Li, *Molecular Evolution* (Sinauer, Sunderland, MA, 1997).
101. M. Cargill et al., *Nature Genet.* 22, 231 (1999).
102. M. K. Halushka et al., *Nature Genet.* 22, 239 (1999).
103. J. Zhang, T. L. Madden, *Genome Res.* 7, 649 (1997).
104. M. Nei, *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987).
105. From the observed coverage of the sequences at each site for each individual, we calculated the probability that a SNP would be detected at the site if it were present. For each level of coverage, there is a binomial sampling of the two homologs for each individual, and a heterozygous site could only be ascertained if both homologs are present, or if two alleles from different individuals are present. With coverage x from a given individual, both homologs are present in the assembly with probability $1 - (1/2)^x$. Even if both homologs are present, the probability that a SNP is detected is < 1 because a fraction of sites failed the quality criteria. Integrating over coverage levels, the binomial sampling, and the quality distribution, we derived an expected number of sites in the genome that were ascertained for polymorphism for each individual. The nucleotide diversity was then the observed number of variable sites divided by the expected number of sites ascertained.
106. M. W. Nachman, V. L. Bauer, S. L. Crowell, C. F. Aquadro, *Genetics* 150, 1133 (1998).
107. D. A. Nickerson et al., *Nature Genet.* 19, 233 (1998); D. A. Nickerson et al., *Genomic Res.* 10, 1532 (2000); L. Jorde et al., *Am. J. Hum. Genet.* 66, 979 (2000); D. G. Wang et al., *Science* 280, 1077 (1998).
108. M. Przeworski, R. R. Hudson, A. Di Rienzo, *Trends Genet.* 16, 296 (2000).
109. S. Tavaré, *Theor. Popul. Biol.* 26, 119 (1984).
110. R. R. Hudson, in *Oxford Surveys in Evolutionary Biology*, D. J. Futuyma, J. D. Antonovics, Eds. (Oxford Univ. Press, Oxford, 1990), vol. 7, pp. 1-44.
111. A. G. Clark et al., *Am. J. Hum. Genet.* 63, 595 (1998).
112. M. Kimura, *The Neutral Theory of Molecular Evolution* (Cambridge Univ. Press, Cambridge, 1983).
113. H. Kaessmann, F. Heissig, A. von Haeseler, S. Paabo, *Nature Genet.* 22, 78 (1999).
114. E. L. Sonnhammer, S. R. Eddy, R. Durbin, *Proteins* 28, 405 (1997).
115. A. Bateman et al., *Nucleic Acids Res.* 28, 263 (2000).
116. Brief description of the methods used to build the Panther classification. First, the June 2000 release of the GenBank NR protein database (excluding sequences annotated as fragments or mutants) was partitioned into clusters using BLASTP. For the clustering, a seed sequence was randomly chosen, and the cluster was defined as all sequences matching the seed to statistical significance (E-value $< 10^{-5}$) and "globally" alignable (the length of the match region must be $> 70\%$ and $< 130\%$ of the length of the seed). If the cluster had more than five mem-

bers, and at least one from a multicellular eukaryote, the cluster was extended. For the extension step, a hidden Markov Model (HMM) was trained for the cluster, using the SAM software package, version 2. The HMM was then scored against GenBank NR (excluding mutants but including fragments for this step), and all sequences scoring better than a specific (NLL-NULL) score were added to the cluster. The HMM was then retrained (with fixed model length) and all sequences in the cluster were aligned to the HMM to produce a multiple sequence alignment. This alignment was assessed by a number of quality measures. If the alignment failed the quality check, the initial cluster was rebuilt around the seed using a more restrictive E-value, followed by extension, alignment, and reassessment. This process was repeated until the alignment quality was good. The multiple alignment and "general" (i.e., describing the entire cluster, or "family") HMM (176) were then used as input into the BETE program (177). BETE calculates a phylogenetic tree for the sequences in the alignment. Functional information about the sequences in each cluster were parsed from SwissProt (178) and GenBank records. "Tree-attribution viewer" software was used by biologist curators to correlate the phylogenetic tree with protein function. Subfamilies were manually defined on the basis of shared function across subtrees, and were named accordingly. HMMs were then built for each subfamily, using information from both the subfamily and family (K. Sjölander, in preparation). Families were also manually named according to the functions contained within them. Finally, all of the families and subfamilies were classified into categories and subcategories based on their molecular functions. The categorization was done by manual review of the family and subfamily names, by examining SwissProt and GenBank records, and by review of the literature as well as resources on the World Wide Web. The current version (2.0) of the Panther molecular function schema has four levels: category, subcategory, family, and subfamily. Protein sequences for whole eukaryotic genomes (for the predicted human proteins and annotated proteins for fly, worm, yeast, and *Arabidopsis*) were scored against the Panther library of family and subfamily HMMs. If the score was significant (the NLL-NULL score cutoff depends on the protein family), the protein was assigned to the family or subfamily function with the most significant score.
- 117. C. P. Ponting, J. Schultz, F. Milpetz, P. Bork, *Nucleic Acids Res.* 27, 229 (1999).
- 118. A. Goffeau et al., *Science* 274, 546, 563 (1996).
- 119. C. elegans Sequencing Consortium, *Science* 282, 2012 (1998).
- 120. S. A. Chervitz et al., *Science* 282, 2022 (1998).
- 121. E. R. Kandel, J. H. Schwartz, T. Jessell, *Principles of Neural Science* (McGraw-Hill, New York, ed. 4, 2000).
- 122. D. A. Goodenough, J. A. Goliger, D. L. Paul, *Annu. Rev. Biochem.* 65, 475 (1996).
- 123. D. G. Wilkinson, *Int. Rev. Cytol.* 196, 177 (2000).
- 124. F. Nakamura, R. G. Kalb, S. M. Strittmatter, *J. Neurobiol.* 44, 219 (2000).
- 125. P. J. Horner, F. H. Gage, *Nature* 407, 963 (2000); P. Casaccia-Bonnel, C. Gu, M. V. Chao, *Adv. Exp. Med. Biol.* 468, 275 (1999).
- 126. S. Wang, B. A. Barres, *Neuron* 27, 197 (2000).
- 127. M. Geppert, T. C. Sudhof, *Annu. Rev. Neurosci.* 21, 75 (1998); J. T. Littleton, H. J. Bellen, *Trends Neurosci.* 18, 177 (1995).
- 128. A. Maximov, T. C. Sudhof, I. Bezprozvanny, *J. Biol. Chem.* 274, 24453 (1999).
- 129. B. Sampo et al., *Proc. Natl. Acad. Sci. U.S.A.* 97, 3666 (2000).
- 130. G. Lemke, *Glia* 7, 263 (1993).
- 131. M. Bernfield et al., *Annu. Rev. Biochem.* 68, 729 (1999).
- 132. N. Perrimon, M. Bernfield, *Nature* 404, 725 (2000).
- 133. U. Lindahl, M. Kusche-Gullberg, L. Kjellen, *J. Biol. Chem.* 273, 24979 (1998).
- 134. J. L. Riechmann et al., *Science* 290, 2105 (2000).
- 135. T. L. Hurskainen, S. Hirohata, M. F. Seldin, S. S. Apte, *J. Biol. Chem.* 274, 25555 (1999).

136. R. A. Black, J. M. White, *Curr. Opin. Cell Biol.* 10, 654 (1998).
137. L. Aravind, V. M. Dixit, E. V. Koonin, *Trends Biochem. Sci.* 24, 47 (1999).
138. A. G. Uren et al., *Mol. Cell* 6, 961 (2000).
139. P. Garcia-Meunier, M. Etienne-Julan, P. Fort, M. Piechaczyk, F. Bonhomme, *Mamm. Genome* 4, 695 (1993).
140. K. Meyer-Siegler et al., *Proc. Natl. Acad. Sci. U.S.A.* 88, 8460 (1991).
141. N. R. Mansur, K. Meyer-Siegler, J. C. Wurzer, M. A. Sirover, *Nucleic Acids Res.* 21, 993 (1993).
142. N. A. Tatton, *Exp. Neurol.* 166, 29 (2000).
143. N. Kenmochi et al., *Genome Res.* 8, 509 (1998).
144. F. W. Chen, Y. A. Ioannou, *Int. Rev. Immunol.* 18, 429 (1999).
145. H. O. Madsen, K. Poulsen, O. Dahl, B. F. Clark, J. P. Hjorth, *Nucleic Acids Res.* 18, 1513 (1990).
146. D. M. Chambers, J. Peters, C. M. Abbott, *Proc. Natl. Acad. Sci. U.S.A.* 95, 4463 (1998); A. Khalyfa, B. M. Carlson, J. A. Carlson, E. Wang, *Dev. Dyn.* 216, 267 (1999).
147. D. Aeschlimann, V. Thomazy, *Connect. Tissue Res.* 41, 1 (2000).
148. P. Munroe et al., *Nature Genet.* 21, 142 (1999); S. M. Wu, W. F. Cheung, D. Frazier, D. W. Stafford, *Science* 254, 1634 (1991); B. Furie et al., *Blood* 93, 1798 (1999).
149. J. W. Kehoe, C. R. Bertozzi, *Chem. Biol.* 7, R57 (2000).
150. T. Pawson, P. Nash, *Genes Dev.* 14, 1027 (2000).
151. A. W. van der Velden, A. A. Thomas, *Int. J. Biochem. Cell Biol.* 31, 87 (1999).
152. C. M. Fraser et al., *Science* 281, 375 (1998); H. Tettelin et al., *Science* 287, 1809 (2000).
153. D. Brett et al., *FEBS Lett.* 474, 83 (2000).
154. H. J. Müller, H. Kern, *Z. Naturforsch. B* 22, 1330 (1967).
155. H. J. Müller, in *Heritage from Mendel*, R. A. Brink, Ed. (Univ. of Wisconsin Press, Madison, WI, 1967), p. 419.
156. J. F. Crow, M. Kimura, *Introduction to Population Genetics Theory* (Harper & Row, New York, 1970).
157. K. Kobayashi et al., *Nature* 394, 388 (1998).
158. A. P. Feinberg, *Curr. Top. Microbiol. Immunol.* 249, 87 (2000).
159. C. A. Collins, C. Guthrie, *Nature Struct. Biol.* 7, 850 (2000).
160. S. R. Eddy, *Curr. Opin. Genet. Dev.* 9, 695 (1999).
161. Q. Wang, J. Khillan, P. Gadue, K. Nishikura, *Science* 290, 1765 (2000).
162. M. Holcik, N. Sonenberg, R. G. Korneluk, *Trends Genet.* 16, 469 (2000).
163. T. A. McKinsey, C. L. Zhang, J. Lu, E. N. Olson, *Nature* 408, 106 (2000).
164. E. Capanna, M. G. M. Romanini, *Caryologia* 24, 471 (1971).
165. J. Maynard Smith, *J. Theor. Biol.* 128, 247 (1987).
166. D. Charlesworth, B. Charlesworth, M. T. Morgan, *Genetics* 141, 1619 (1995).
167. J. E. Bailey, *Nature Biotechnol.* 17, 616 (1999).
168. R. Maleszka, H. G. de Couet, G. L. Miklos, *Proc. Natl. Acad. Sci. U.S.A.* 95, 3731 (1998).
169. G. L. Miklos, *J. Neurobiol.* 24, 842 (1993).
170. J. P. Crutchfield, K. Young, *Phys. Rev. Lett.* 63, 105 (1989); M. Gell-Mann, S. Lloyd, *Complexity* 2, 44 (1996).
171. A. L. Barabasi, R. Albert, *Science* 286, 509 (1999).
172. E. Colucci-Guyon et al., *Cell* 79, 679 (1994).
173. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989).
174. B. Ewing, P. Green, *Genome Res.* 8, 186 (1998); B. Ewing, L. Hillier, M. C. Wendt, P. Green, *Genome Res.* 8, 175 (1998).
175. E. S. Lander, M. S. Waterman, *Genomics* 2, 231 (1988).
176. A. Krogh, K. Sjölander, *J. Mol. Biol.* 235, 1501 (1994).
177. K. Sjölander, *Proc. Int. Soc. Mol. Biol.* 6, 165 (1998).
178. A. Bairoch, R. Apweiler, *Nucleic Acids Res.* 28, 45 (2000).
179. GO, available at www.geneontology.org/.
180. R. L. Tatusov, M. Y. Galperin, D. A. Natale, E. V. Koonin, *Nucleic Acids Res.* 28, 33 (2000).
181. We thank E. Eichler and J. L. Goldstein for many helpful discussions and critical reading of the manuscript, and A. Caplan for advice and encouragement. We also thank T. Hein, D. Lucas, G. Edwards, and the Celera IT staff for outstanding computational support. The cost of this project was underwritten by the Celera Genomics Group of the Applera Corporation. We thank the Board of Directors of Applera Corporation: J. F. Abely Jr. (retired), R. H. Ayers, J.-L. Bélingard, R. H. Hayes, A. J. Levine, T. E. Martin, C. W. Slayman, O. R. Smith, G. C. St. Laurent Jr., and J. R. Tobin for their vision, enthusiasm, and unwavering support and T. L. White for leadership and advice. Data availability: The genome sequence and additional supporting information are available to academic scientists at the Web site (www.celera.com). Instructions for obtaining a DVD of the genome sequence can be obtained through the Web site. For commercial scientists wishing to verify the results presented here, the genome data are available upon signing a Material Transfer Agreement, which can also be found on the Web site.

5 December 2000; accepted 19 January 2001

Science

Functional Genomics Website

- Links to breaking news in genomics and biotech from *Science*, *ScienceNOW*, and other sources.
- Pointers to classic papers, reviews, and new research, organized by subject relevant to the post-genomics world.
- *Science's* genome special issues.
- Collections of Web resources in genomics and post-genomics, including special pages on model organisms, educational resources, and genome maps.
- A central node of news, information, and links to the biotech business.

www.sciencegenomics.org

C

This Page Blank (uspto)

>XM_093852 ACCESSION:XM_093852 NID: gi 18556797 ref XM_093852.1
Homo sapiens similar to epidermis specific serine
protease (LOC166414), mRNA
Length = 1095

Score = 507 bits (1291), Expect = e-141
Identities = 242/244 (99%), Positives = 242/244 (99%), Gaps = 1/244 (0%)
Frame = +1

Query: 1 MGPAGCAFTLLLLLGISVCGQPVSRRVVGQDAAAGRWPQVSLHFDHNFYGGSLVSE 60
MGPAGCAFTLLLLLGISVCGQPVSRRVVGQDAAAGRWPQVSLHFDHNFY GGSLVSE
Sbjct: 1 MGPAGCAFTLLLLLGISVCGQPVSRRVVGQDAAAGRWPQVSLHFDHNFICGGSVSE 180

Query: 61 RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRVKYYVSKIVIHHPKYQD TTAD-ALLKL 119
RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRVKYYVSKIVIHHPKYQD TTAD ALLKL
Sbjct: 181 RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRVKYYVSKIVIHHPKYQD TTADVALLKL 360

Query: 120 SSQVTFTSAILPICLPSVTKQLAIPFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA 179
SSQVTFTSAILPICLPSVTKQLAIPFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA
Sbjct: 361 SSQVTFTSAILPICLPSVTKQLAIPFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA 540

Query: 180 CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIQTGVVSW 239
CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIQTGVVSW
Sbjct: 541 CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIQTGVVSW 720

Query: 240 GLEC 243
GLEC
Sbjct: 721 GLEC 732

D

This Page Blank (uspto)

>AX360076 ACCESSION:AX360076 NID:18675702 Homo sapiens Sequence 32
from Patent WO0200860. gbPatent
Length = 987

Score = 675 bits (1724), Expect = 0.0
Identities = 325/328 (99%), Positives = 325/328 (99%), Gaps = 2/328 (0%)
Frame = +1

Query: 1 MGPAGCAFTLLLLLGISVCGQPVYSSRVVGGQDAAAGRWPQVSLHFDHNFIIYGGSLVSE 60
MGPAGCAFTLLLLLGISVCGQPVYSSRVVGGQDAAAGRWPQVSLHFDHNFII GGSLVSE
Sbjct: 1 MGPAGCAFTLLLLLGISVCGQPVYSSRVVGGQDAAAGRWPQVSLHFDHNFICGGSLVSE 180

Query: 61 RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRKVKYVSKIVIHHPKYQDTTAD-ALLKL 119
RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRKVKYVSKIVIHHPKYQDTTAD ALLKL
Sbjct: 181 RLILTAAHCIQPTWTTFSYTVWLGSITVGDSRKRKVKYVSKIVIHHPKYQDTTADVALLKL 360

Query: 120 SSQVTFTSAILPICLPSVTKQLAIPFFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA 179
SSQVTFTSAILPICLPSVTKQLAIPFFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA
Sbjct: 361 SSQVTFTSAILPICLPSVTKQLAIPFFCWVTGWGKVKESDRDYHSALQEAEPPIIDRQA 540

Query: 180 CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIIQTGVVSW 239
CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIIQTGVVSW
Sbjct: 541 CEQLYNPIGIFLPALEPVIKEDKICAGDTQNMKDSCKGDSGGPLSCHIDGVWIIQTGVVSW 720

Query: 240 GLECGKSLPGVYTNVIYYQKWINATISRANNLDFSDFLFPIVLLSLALL-PSCAFGPNTI 298
GLECGKSLPGVYTNVIYYQKWINATISRANNLDFSDFLFPIVLLSLALL PSCAFGPNTI
Sbjct: 721 GLECGKSLPGVYTNVIYYQKWINATISRANNLDFSDFLFPIVLLSLALLRPSCAFGPNTI 900

Query: 299 HRVGTVAEAVACIQGWEENAWRFSPRGR 326
HRVGTVAEAVACIQGWEENAWRFSPRGR
Sbjct: 901 HRVGTVAEAVACIQGWEENAWRFSPRGR 984

Presentation: Image: Français

1 of 6

[View Images](#)

PUBLISHED INTERNATIONAL APPLICATION

- (11) **WO 02/00860** (13) **A2**
 (21) **PCT/US01/20171**
 (22) **26 June 2001 (26.06.2001)**
 (25) **ENG** (26) **ENG**
 (31) **60/214,047** (32) **26 June 2000 (26.06.2000)** **US**
 (43) **03 January 2002 (03.01.2002)**
 (51)⁷ **C12N 9/00**
 (54) **NOVEL PROTEASES**
 (71) **SUGEN, INC.** 230 East Grand Avenue, South San Francisco, CA 94080-4811; (US). [US/US].(for all designated States except US)
 (72)(75) **PLOWMAN, Gregory** 35 Winding Way, San Carlos, CA 94070; (US)
 [US/US].**WHYTE, David** **WHYTE, David**; (US)
 [US/US].**SUDARSANAM, Sucha** **SUDARSANAM, Sucha**; (US)
 [US/US].**MANNING, Gerard** **MANNING, Gerard**; (US)
 [US/IE].**CAENEPEEL, Sean** **CAENEPEEL, Sean**; (US)
 [US/US].**CHARYDCZAK, Glen** **CHARYDCZAK, Glen**; (US)
 [US/US].
 (74) **BURROUS, Beth, A.** Foley & Lardner, Washington Harbour, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109; (US).
 (81)
 AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW
 (84)
 ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG)

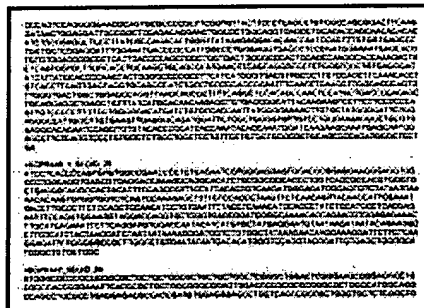
[For information on time limits for entry into the national phase please click here](#)

Published *without international search report and to be republished*

upon receipt of that report

Abstract

The present invention relates to protease polypeptides, nucleotide sequences encoding the protease polypeptides, as well as various products and methods useful for the diagnosis and treatment of various protease-related diseases and conditions.



Presentation:

Basic



Image:

Small



Français



1 of 6

E

This Page Blank (uspto)



US005445934A

United States Patent [19]

Fodor et al.

[11] **Patent Number:** 5,445,934[45] **Date of Patent:** Aug. 29, 1995[54] **ARRAY OF OLIGONUCLEOTIDES ON A SOLID SUBSTRATE**[75] **Inventors:** Stephen P. A. Fodor, Palo Alto, Calif.; Michael C. Pirrung, Durham, N.C.; J. Leighton Read, Palo Alto; Lubert Stryer, Stanford, both of Calif.[73] **Assignee:** Affymax Technologies N.V., Curacao, Netherlands Antilles[21] **Appl. No.:** 954,646[22] **Filed:** Sep. 30, 1992**Related U.S. Application Data**

[60] Division of Ser. No. 850,356, Mar. 12, 1992, which is a division of Ser. No. 492,462, Mar. 7, 1990, Pat. No. 5,143,854, which is a continuation-in-part of Ser. No. 362,901, Jun. 7, 1989, abandoned.

[51] **Int. Cl.⁶** C12Q 1/68; G01N 33/543[52] **U.S. Cl.** 435/6; 435/7.92; 435/969; 435/973; 436/518; 436/527; 436/807; 436/809; 536/25.3; 428/426; 428/532[58] **Field of Search** 435/7.92, 7.94, 7.95, 435/969, 973, 6; 536/25.3-25.34; 935/88; 436/518, 527, 807, 809; 530/334; 427/2; 428/436, 532[56] **References Cited****U.S. PATENT DOCUMENTS**

4,562,157 12/1985 Lowe 435/291
 4,689,405 8/1987 Frank et al. 536/27
 4,728,591 3/1988 Clark et al. 430/5
 4,886,741 12/1989 Schwartz 435/5
 4,888,278 12/1989 Singer et al. 435/6

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

0328256 8/1989 European Pat. Off. B01J 20/32
 WO89/10977 11/1989 WIPO C12Q 1/68
 WO90/03382 5/1990 WIPO C07H 21/00

OTHER PUBLICATIONS

BioRad Chromatography, Electrophoresis, Immunochemistry, Molecular Biology, HPLC Catalogue M 1987 p. 182.

Hames & Heggins (ed). Nucleic Acid Hybridization: A Practical Approach (1985) IRL Press Oxford England. Science, 253, p. 1489, Sep. 27, 1991 "Will DNA Chip Speed Genome Initiative?"

Khrapko et al., FEB, 256, pp. 118-122, Oct. 1989 "An oligonucleotide hybridization approach to DNA sequencing".

Mirzabekov, Tibtech, 12, pp. 27-32, Jan. 1994 "DNA sequencing by hybridization—a megasequencing method and a diagnostic tool?"

Southern et al., Genomics, 13, pp. 1008-1017, 1992 "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation Using Experimental Models".

Haridasan et al., "Peptide Synthesis Using Photolyti- (List continued on next page.)

Primary Examiner—Esther M. Kepplinger*Assistant Examiner*—Lora M. Green*Attorney, Agent, or Firm*—Townsend and Townsend Khourie and Crew

[57]

ABSTRACT

A method and apparatus for preparation of a substrate containing a plurality of sequences. Photoremovable groups are attached to a surface of a substrate. Selected regions of the substrate are exposed to light so as to activate the selected areas. A monomer, also containing a photoremovable group, is provided to the substrate to bind at the selected areas. The process is repeated using a variety of monomers such as amino acids until sequences of a desired length are obtained. Detection methods and apparatus are also disclosed.

10 Claims, 20 Drawing Sheets

U.S. PATENT DOCUMENTS

4,923,901	5/1990	Koester et al.	536/25.3 X
4,973,493	11/1990	Guire	427/2
5,079,600	1/1992	Schnur et al.	357/4
5,143,854	9/1992	Pirrung et al.	436/518
5,202,231	4/1993	Drmanac et al.	435/6

OTHER PUBLICATIONS

cally Cleavable 2-Nitrobenzyloxycarbonyl Protecting Group," *Proc. Indian Natl. Sci. Acad., Part A* (1987) 53:717-728.

Sze/McGillis, *VLSI Technology*, Chapter 7, pp. 267-301, McGraw-Hill, 1983.

Geysen et al., "Strategies for epitope analysis using peptide synthesis," *J. Immunol. Meth.* (1987) 102:259-274.

Furka et al., "More Peptides by Less Labor," Abstract No. 288 from *Xth International Symposium on Medicinal Chemistry*, Budapest, Hungary, Aug. 15-19, 1988.

Ohtsuka et al., "Studies on transfer ribonucleic acids and related compounds. IX(1) Ribooligonucleotide synthesis . . . 2'-hydroxyl group," *Nucleic Acids Research* (1974) 1:1351-1357.

Kleinfeld et al., "Controlled Outgrowth of Dissociated Neurons on Patterned Substrates," *J. of Neuroscience*, 8(11):4098-4120, Nov. 1988.

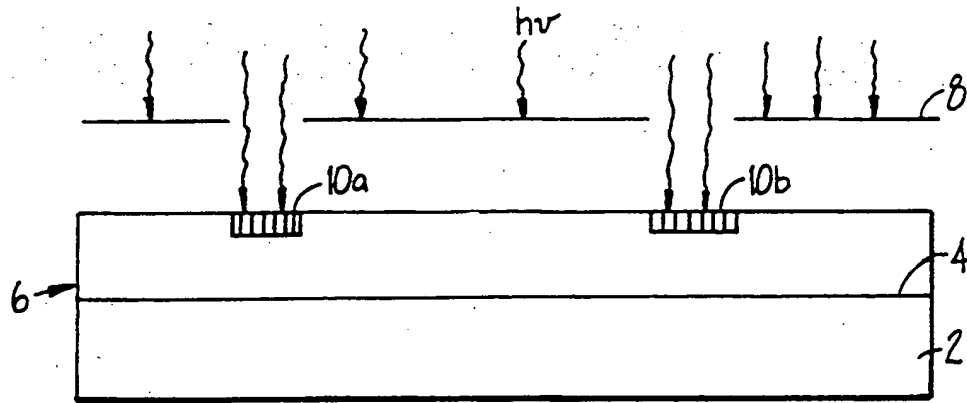


FIG. 1.

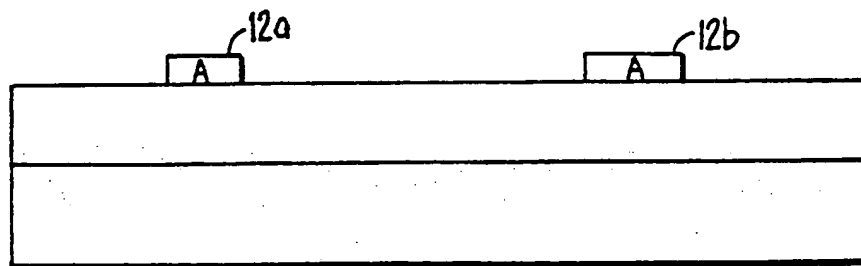


FIG. 2.

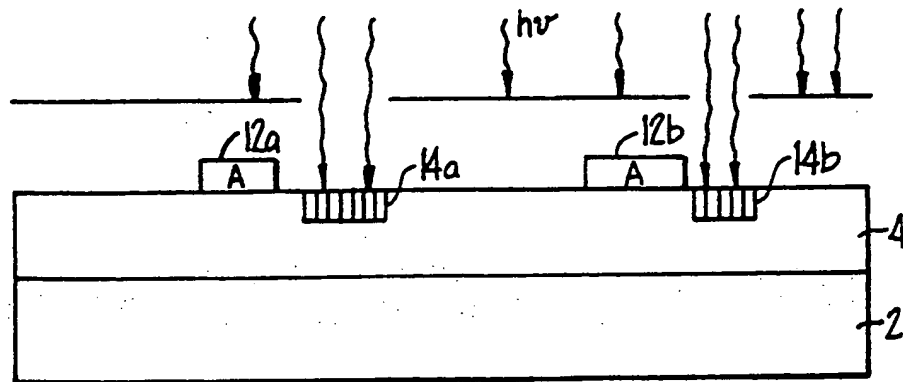


FIG. 3.

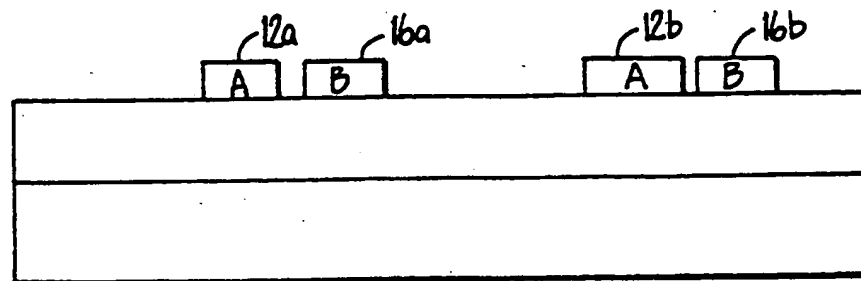


FIG. 4.

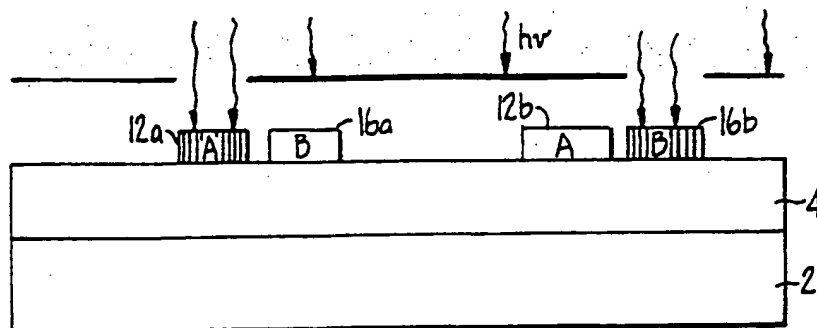


FIG. 5.

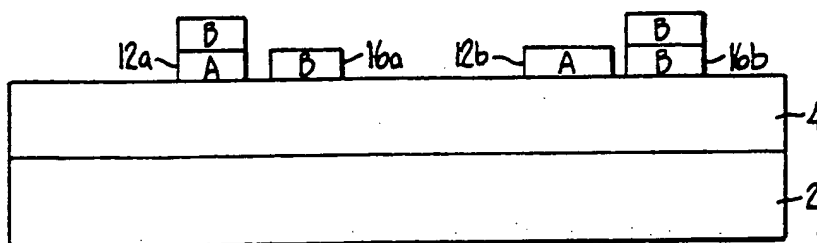


FIG. 6.

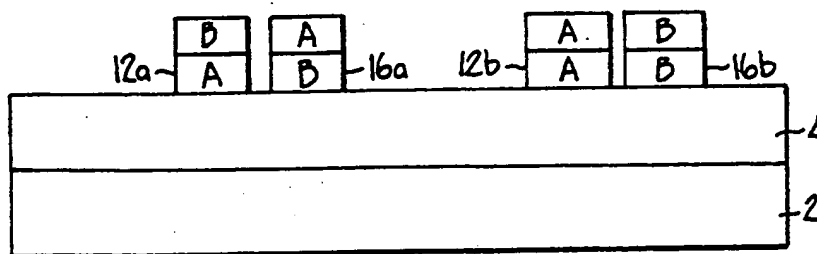


FIG. 7.

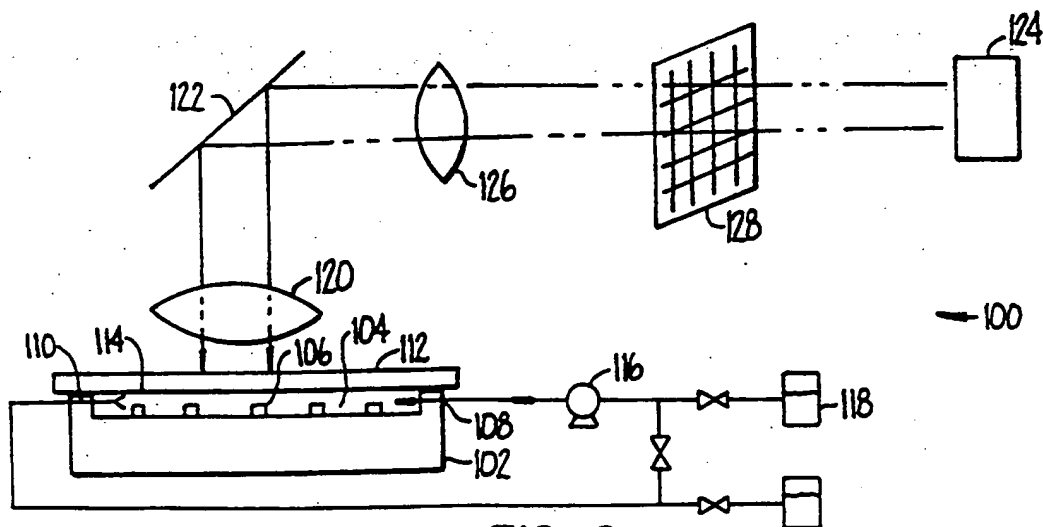


FIG. 8a.

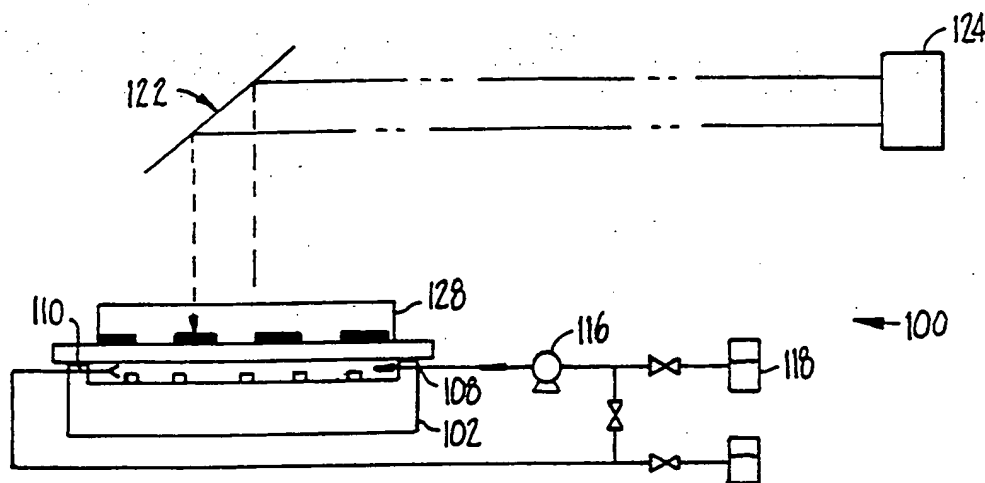


FIG. 8b.

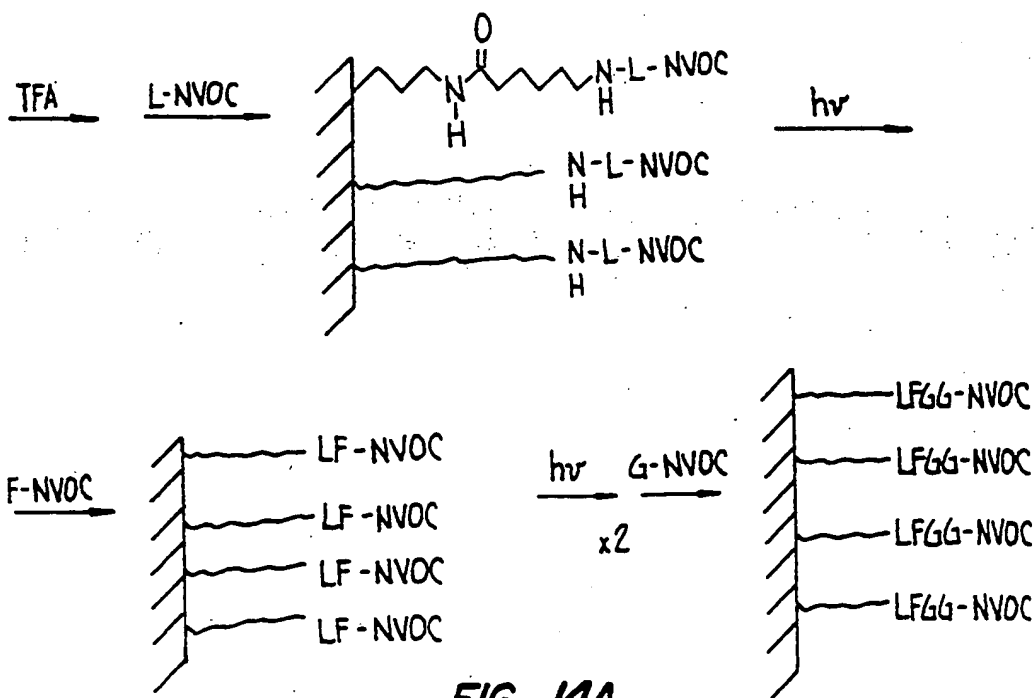
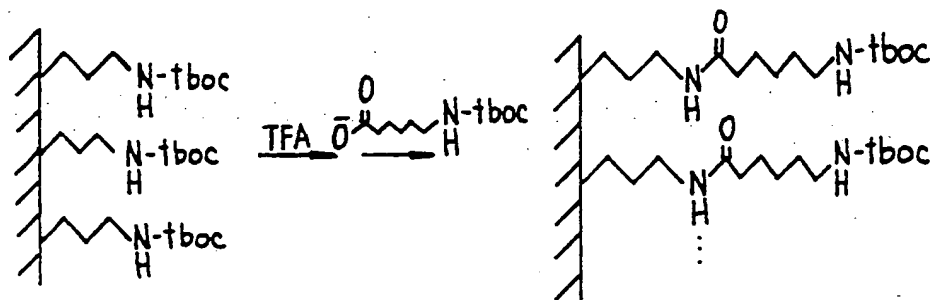


FIG. 14A.

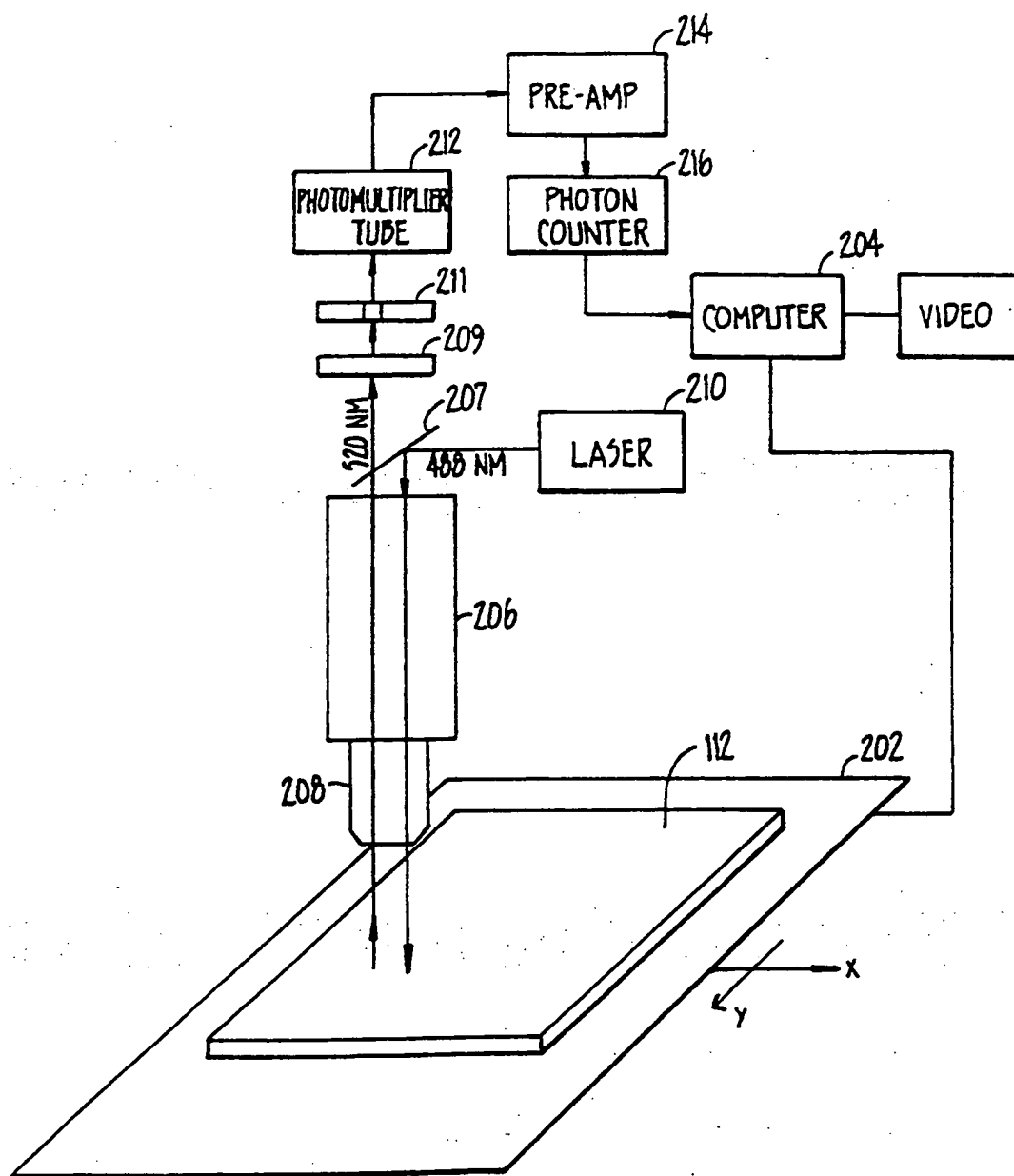


FIG. 9.

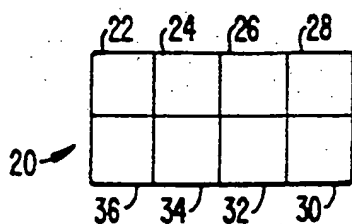


FIG. 10A.

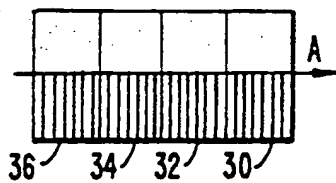


FIG. 10B.

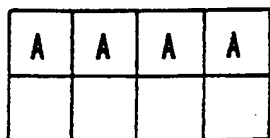


FIG. 10C.

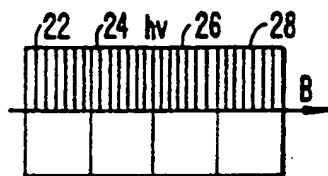


FIG. 10D.

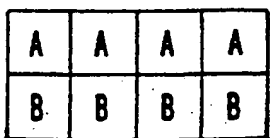


FIG. 10E.

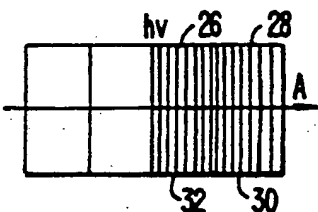


FIG. 10F.

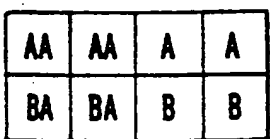


FIG. 10G.

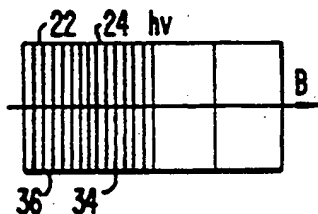


FIG. 10H.

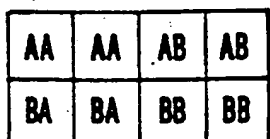


FIG. 10I.

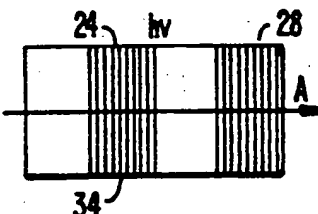


FIG. 10J.

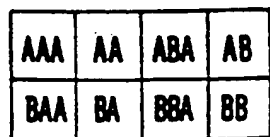


FIG. 10K.

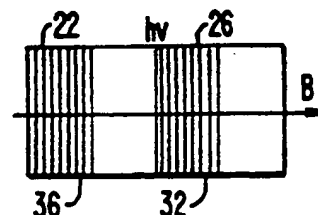


FIG. 10L.

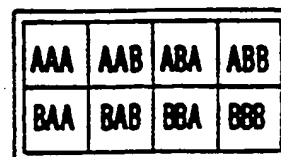


FIG. 10M.

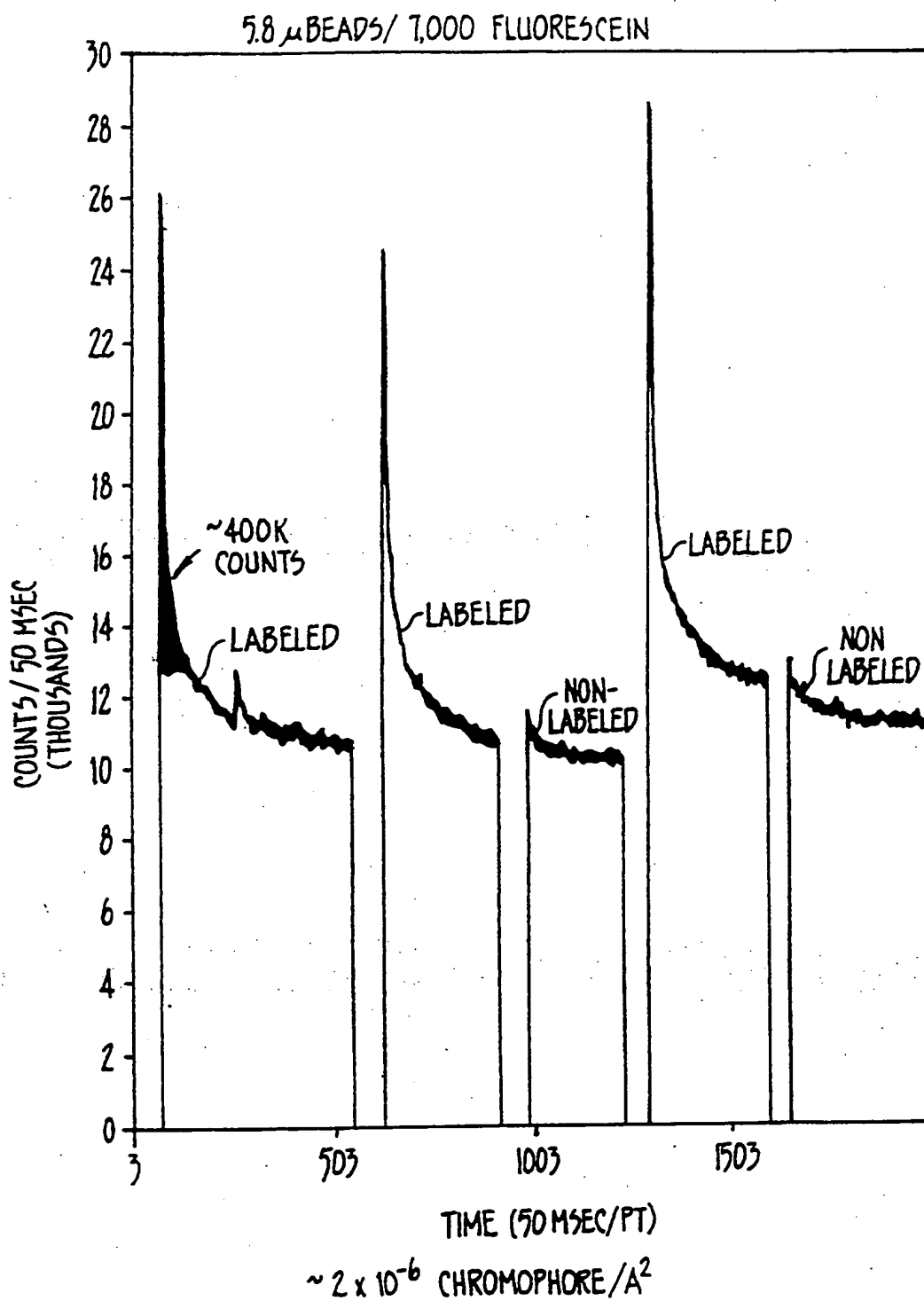


FIG. IIA.

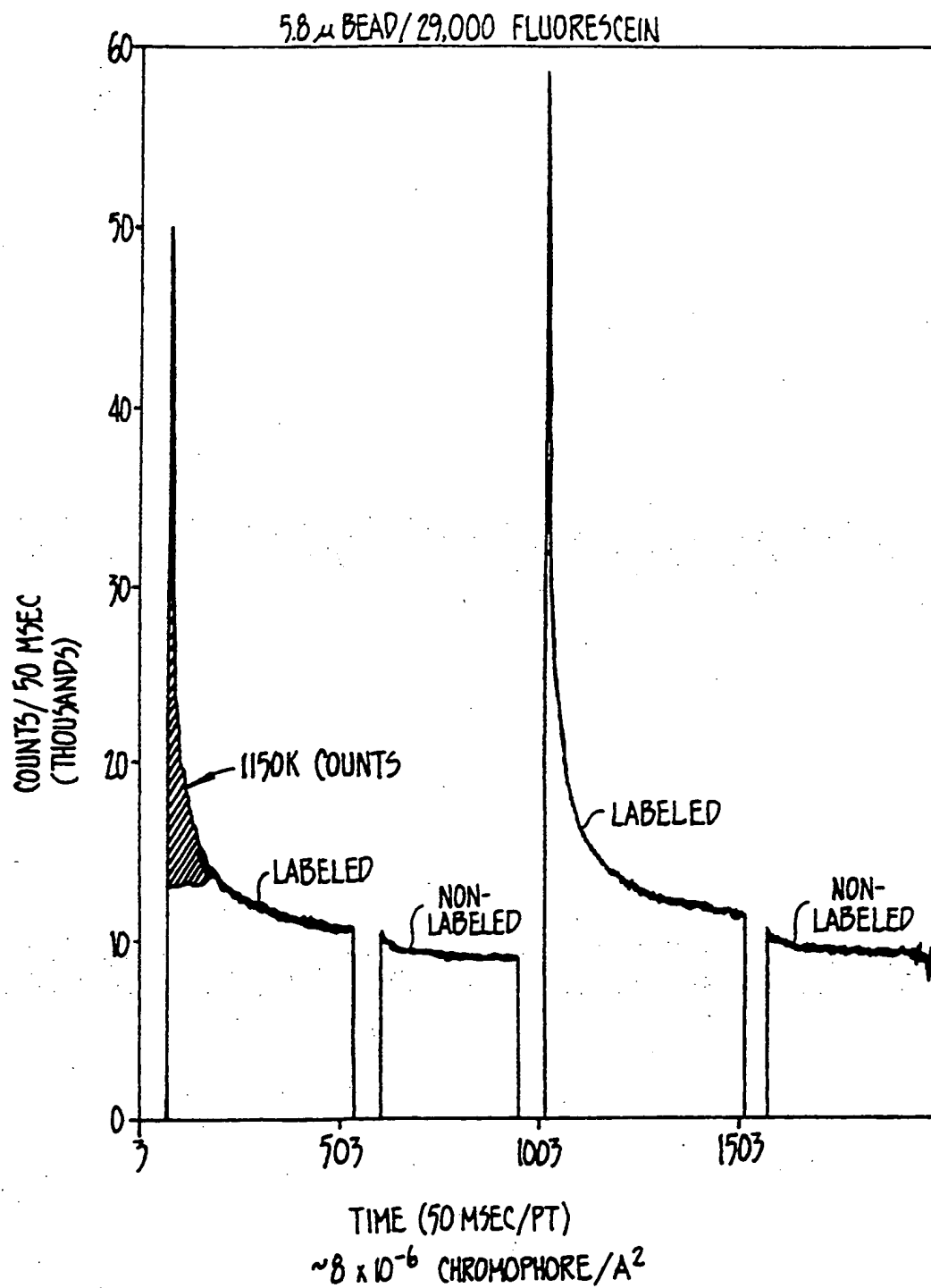


FIG. IIB.

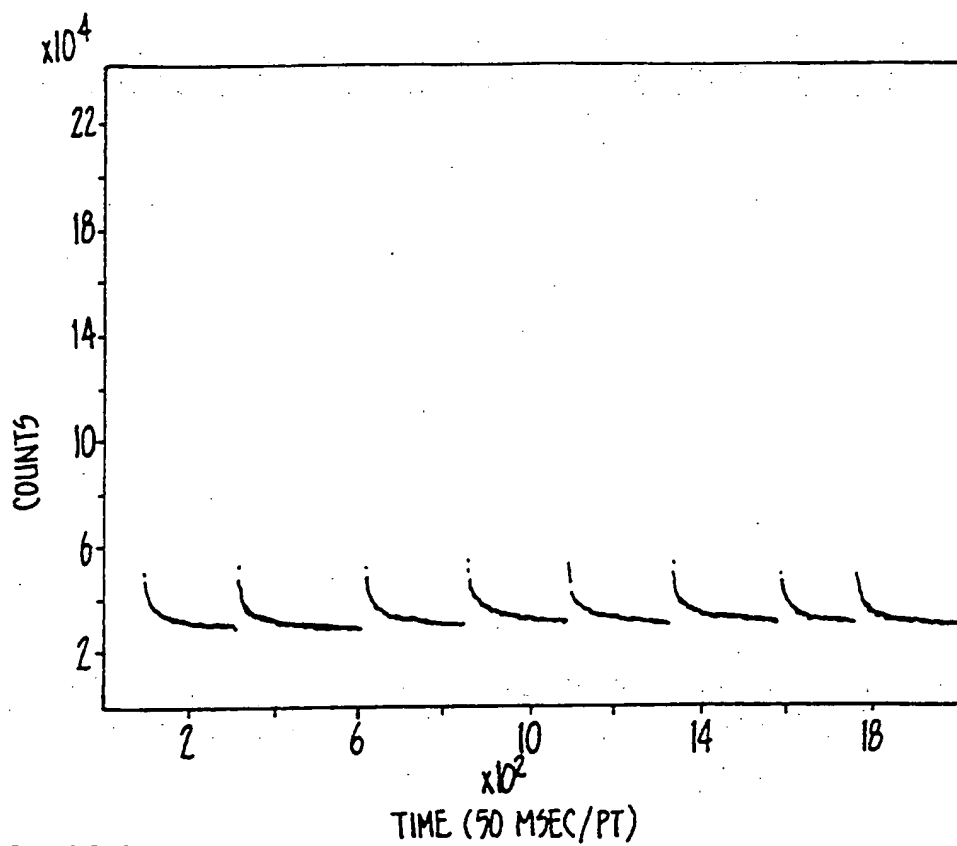


FIG. 12A.

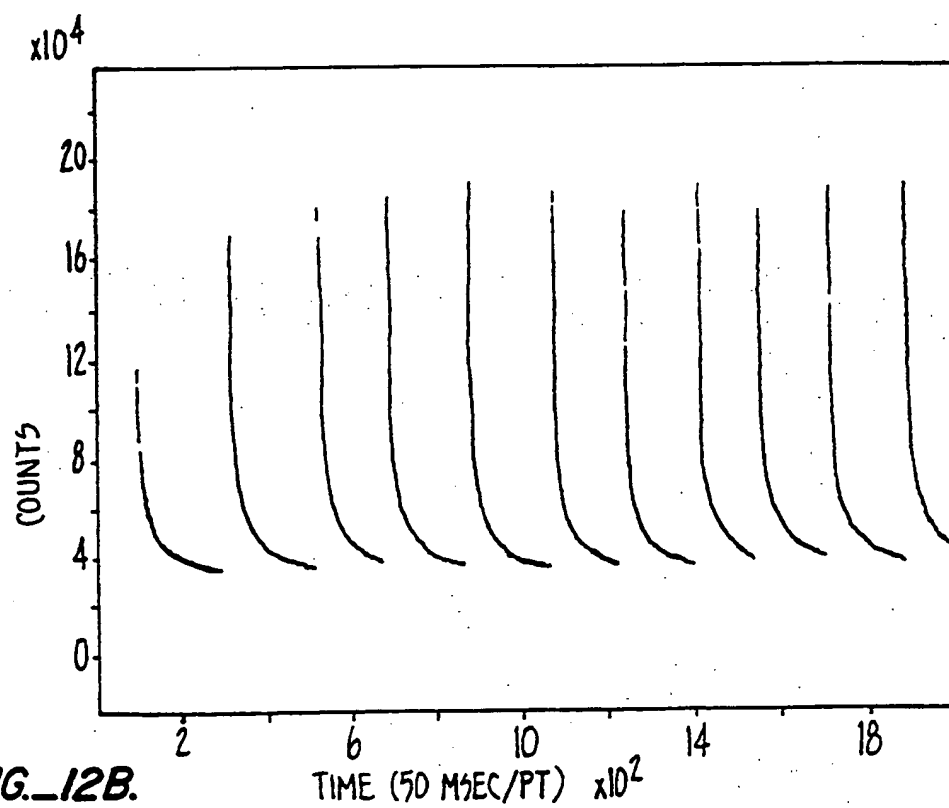
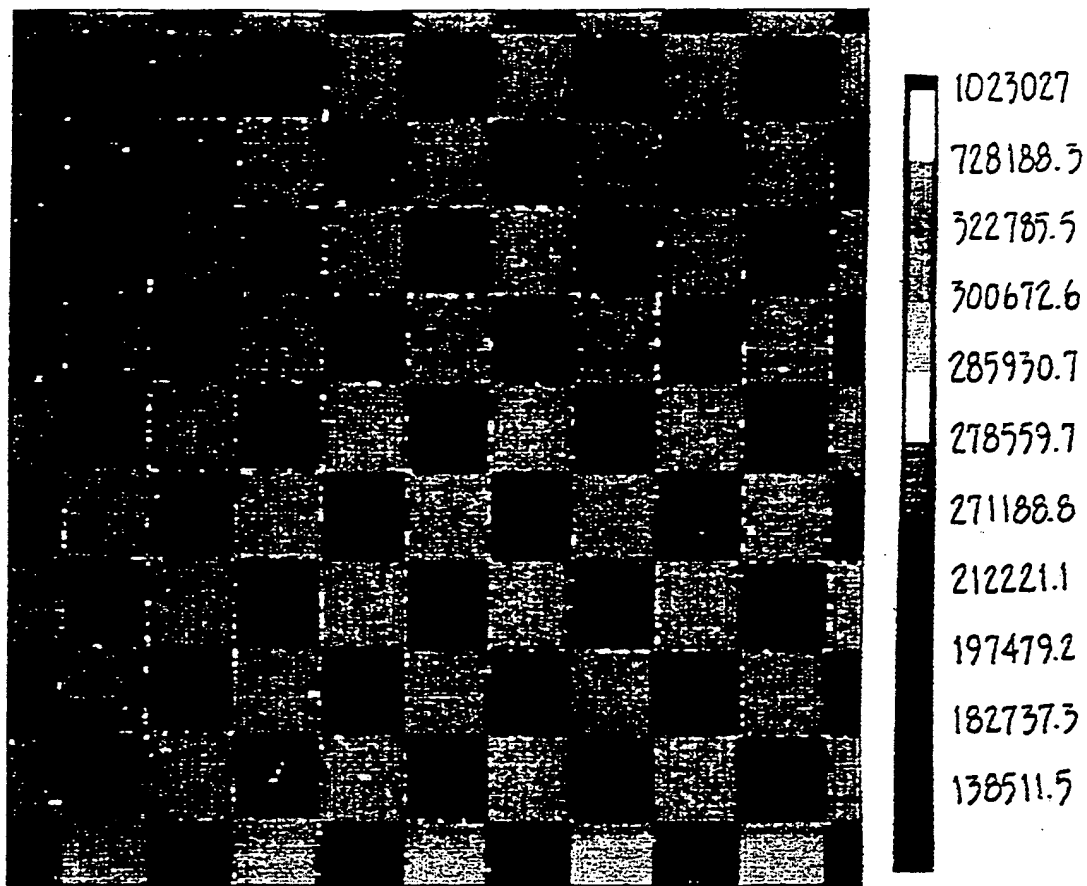
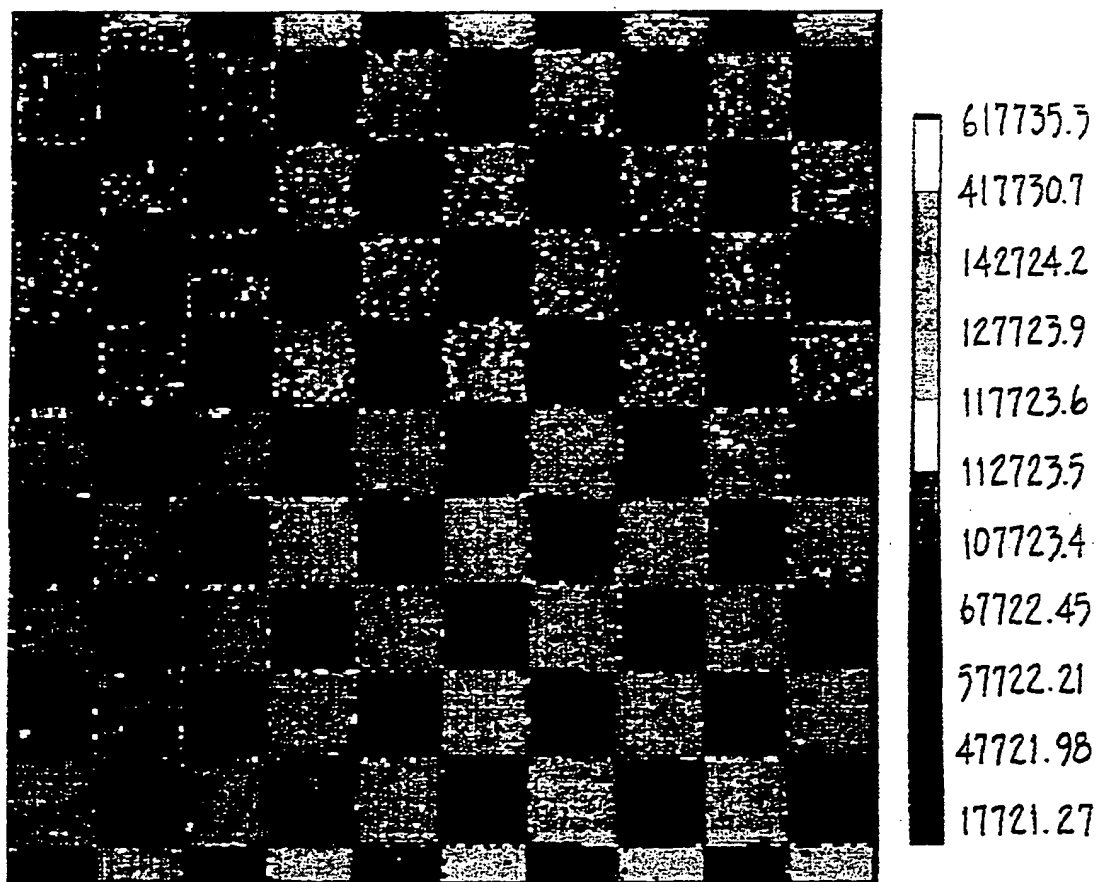


FIG. 12B.



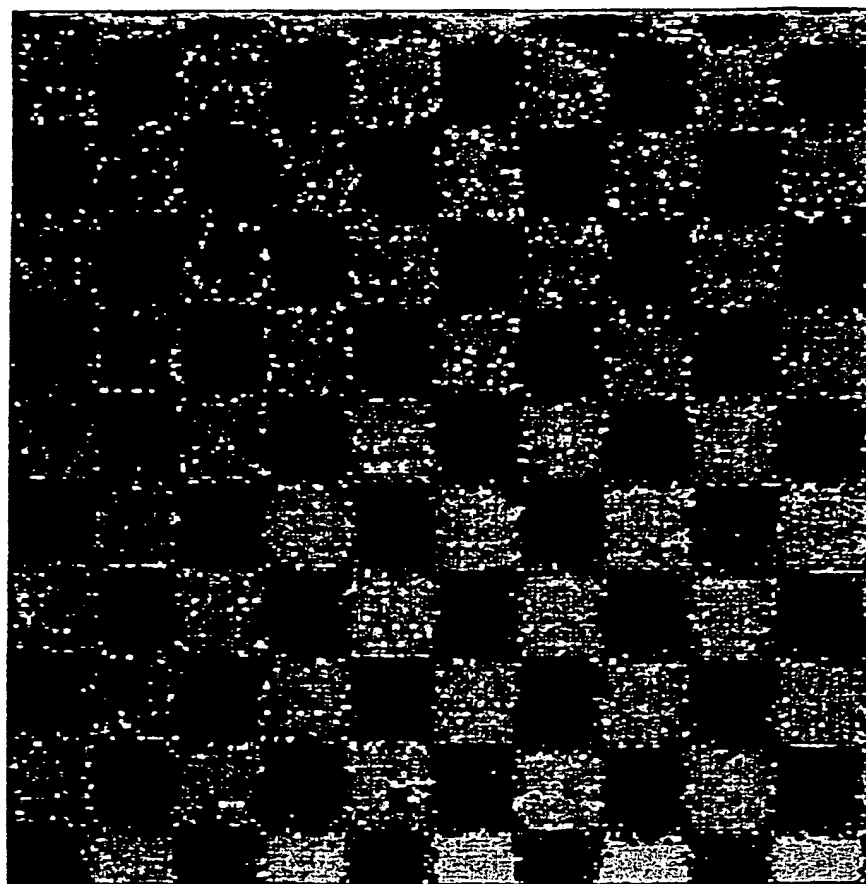
MEAN: 285930.7
VAR: 2.173242E+10
 σ : 147419.2

FIG. 13A.



MEAN: 117723.6
VAR: 1.000047E+10
 σ : 100002.3

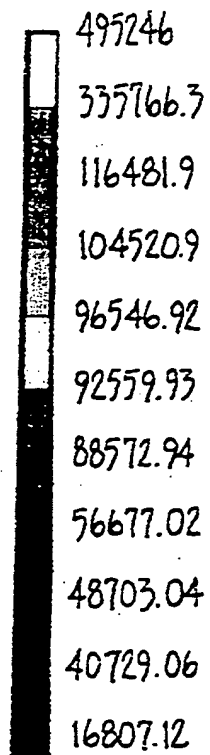
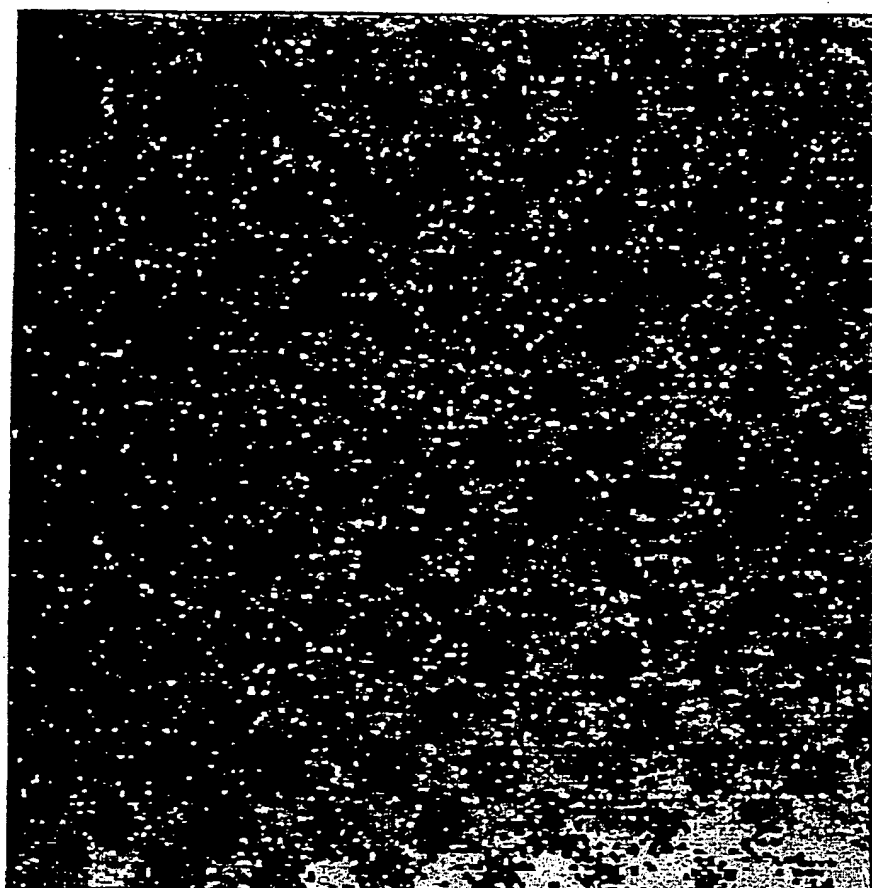
FIG. 13B.



- 552484.3
373317.4
126963
113525.5
104567.2
100000
95608.83
59775.46
50017.12
41858.78
14983.75

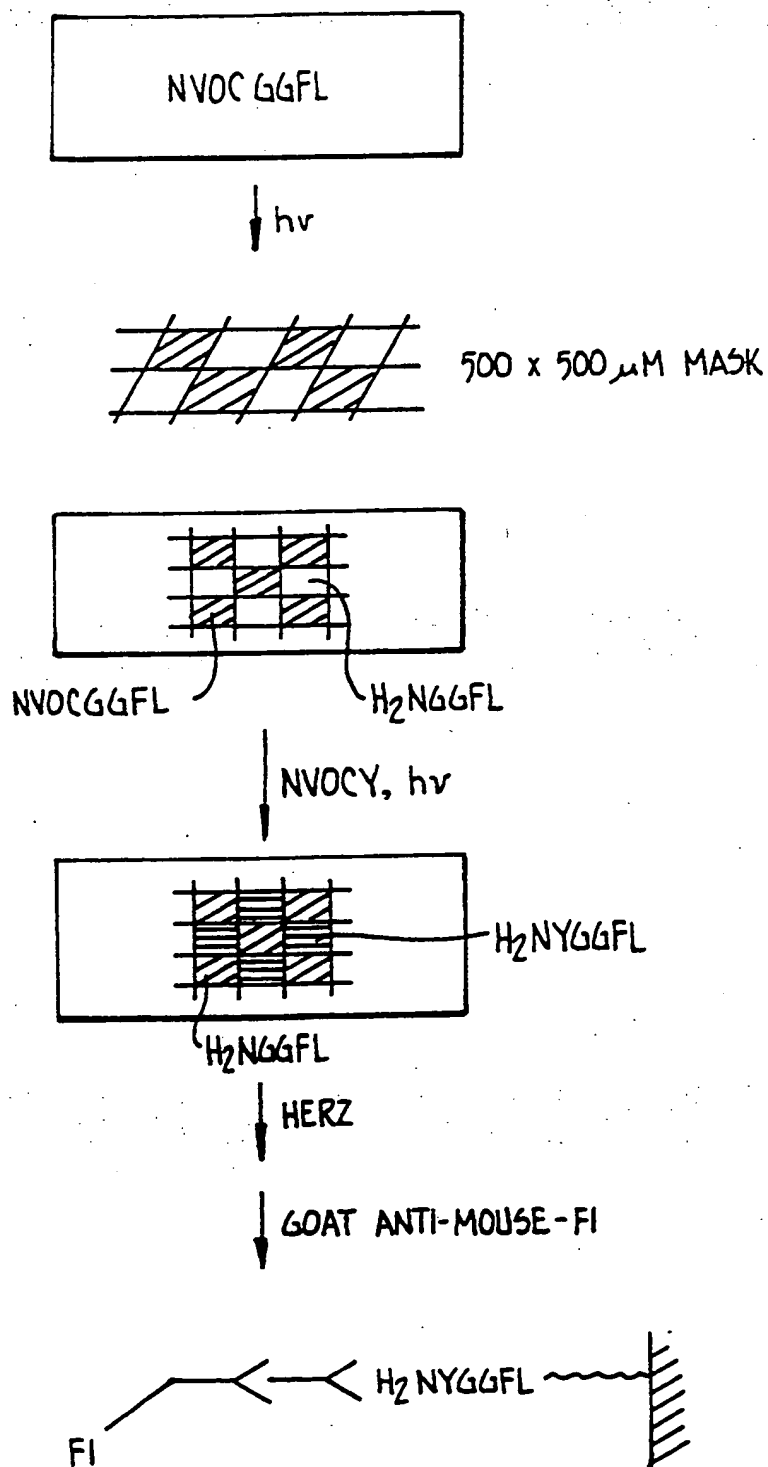
MEAN: 104567.2
VAR: 8.025189E+09
σ: 89585.42

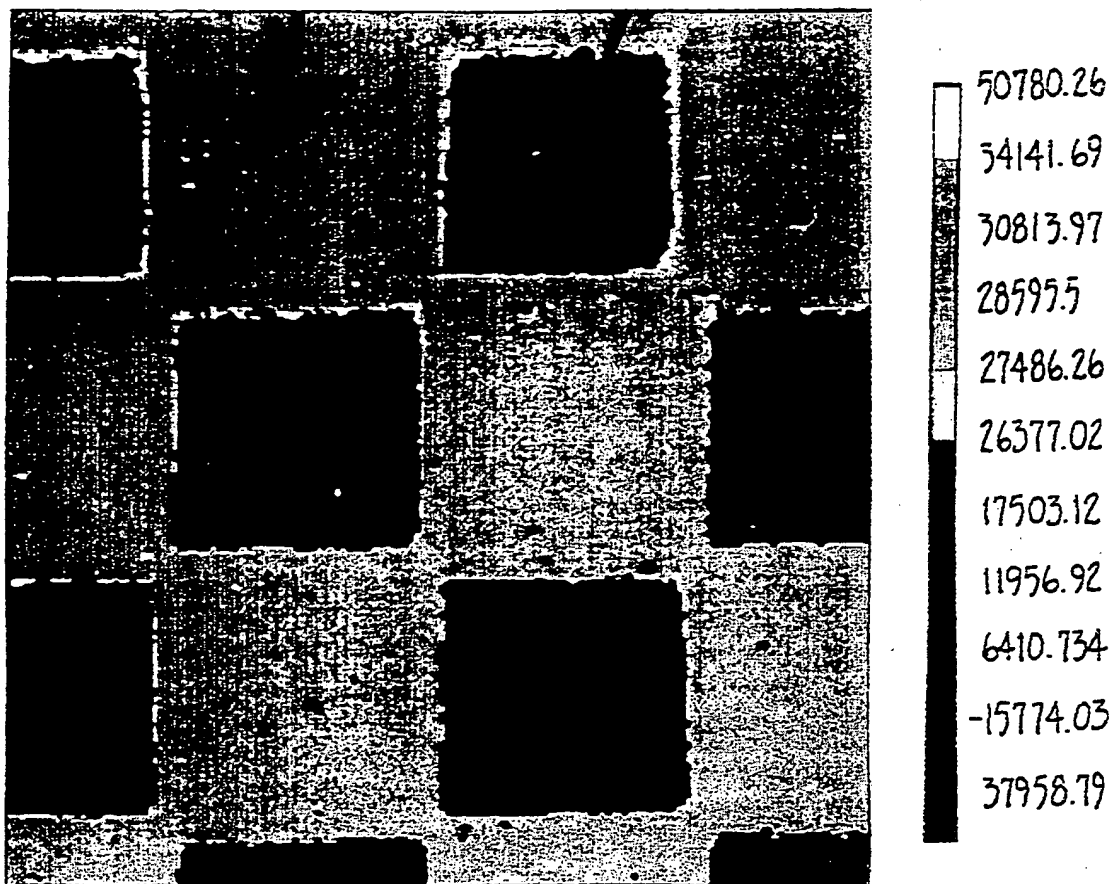
FIG. 13C.



MEAN: 96546.92
VAR: 6.358437E+09
 σ : 79739.6

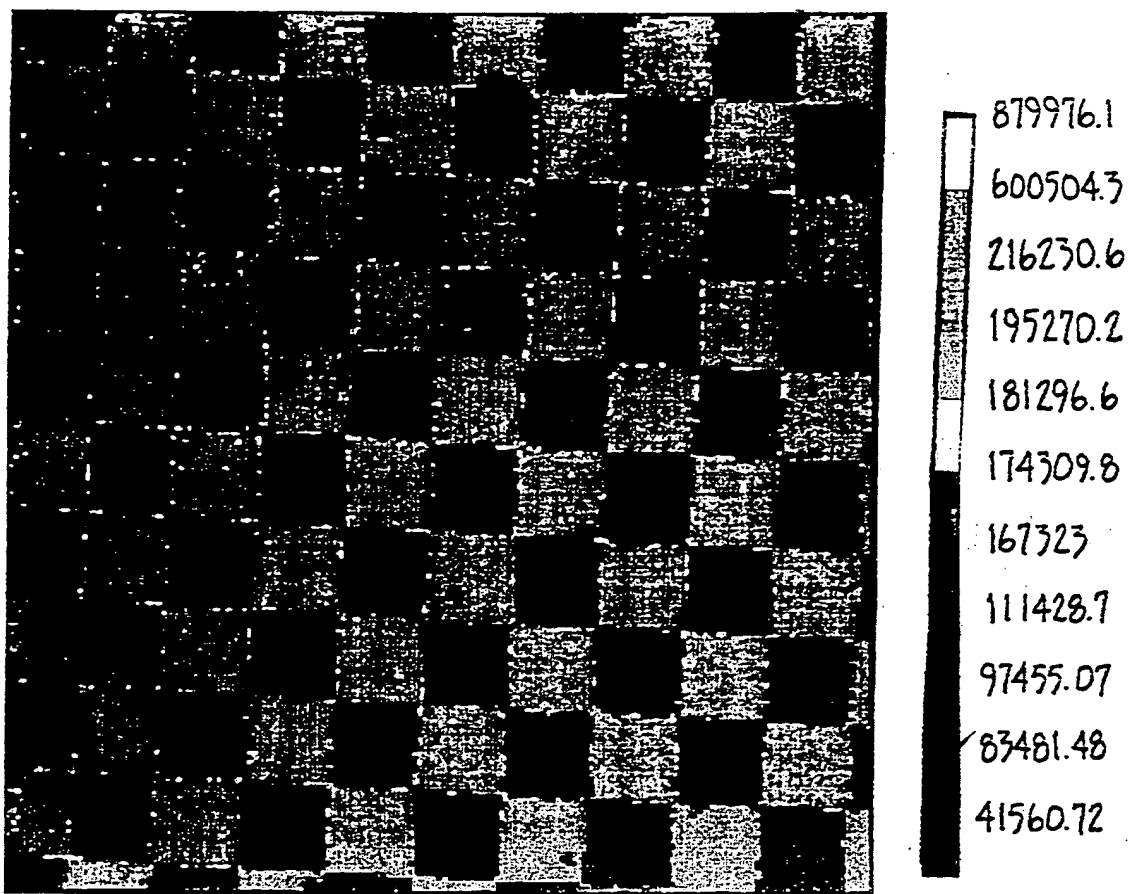
FIG. 13D.

**FIG. 14B.**



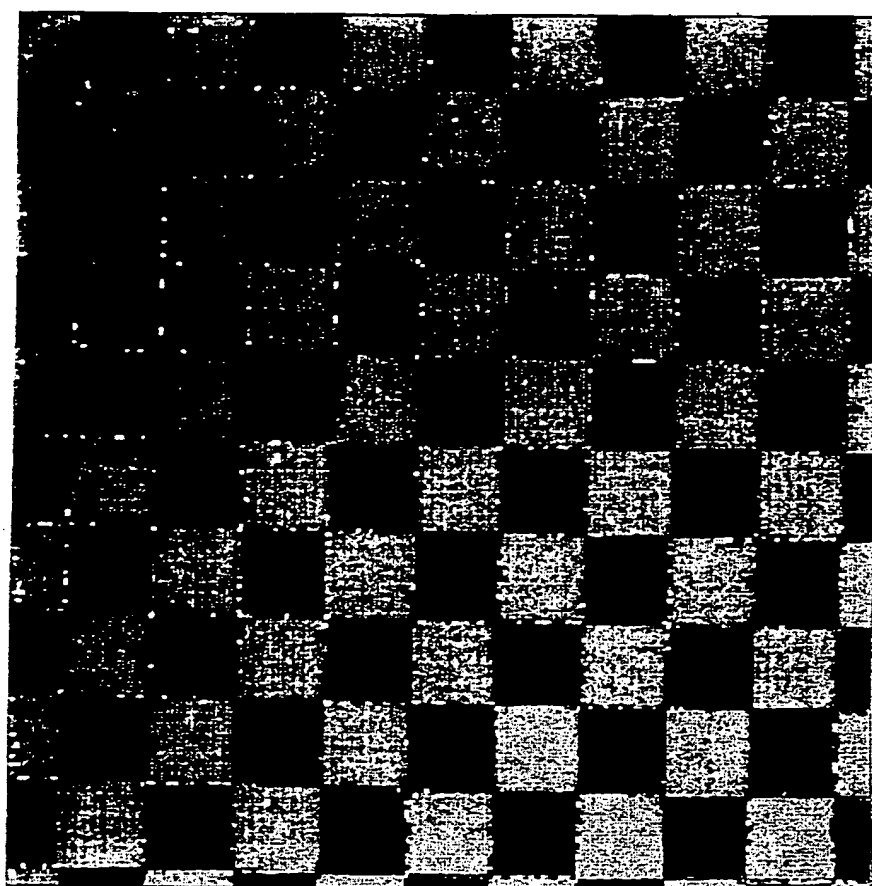
MEAN: 28595.5
VAR: 4.921637E+06
 σ : 22184.76

FIG. 15A.



MEAN: 181296.6
VAR: 1.952612E+10
σ: 139735.9

FIG. 15B.



676588

428583.8

142577.9

126977.5

116577.3

111377.2

106177.1

64576.25

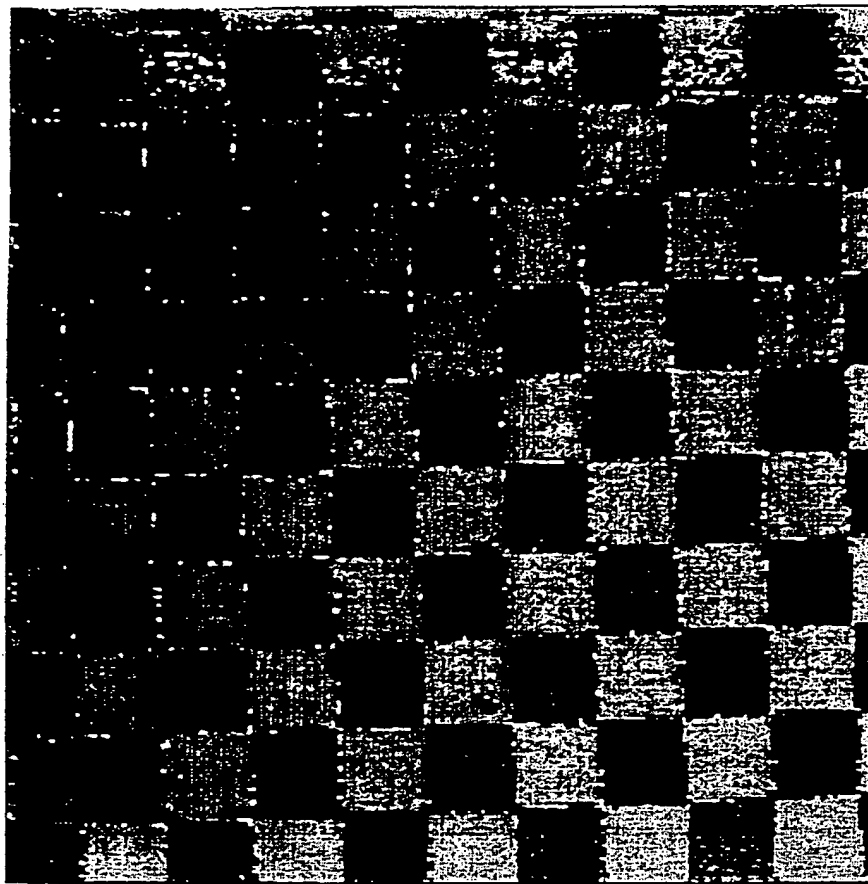
54176.03

43775.82

12575.18

MEAN: 116577.3
VAR: 1.081645E+10
σ: 104002.1

FIG. 16.



667348.3
453053
158397
142324.9
131610.1
126252.7
120895.3
78036.29
67321.52
56606.77
24462.47

MEAN: 131610.1
VAR: 1.148062E+10
 σ : 107147.6

FIG. 17.

P	A	S	G	
<u>L</u> P GFL	<u>L</u> A GFL	<u>L</u> S GFL	<u>L</u> G GFL	L
<u>F</u> P GFL	<u>F</u> A GFL	<u>F</u> S GFL	<u>F</u> G GFL	F
<u>W</u> P GFL	<u>W</u> A GFL	<u>W</u> S GFL	<u>W</u> G GFL	W
<u>Y</u> P GFL	<u>Y</u> A GFL	<u>Y</u> S GFL	<u>Y</u> G GFL	Y

L SET

FIG. 18A.

p	a	s	G	
<u>Y</u> p GFL	<u>Y</u> a GFL	<u>Y</u> s GFL	<u>Y</u> G GFL	Y
<u>f</u> p GFL	<u>f</u> a GFL	<u>f</u> s GFL	<u>f</u> G GFL	f
<u>w</u> p GFL	<u>w</u> a GFL	<u>w</u> s GFL	<u>w</u> G GFL	w
<u>y</u> p GFL	<u>y</u> a GFL	<u>y</u> s GFL	<u>y</u> G GFL	y

D SET

FIG. 18B.

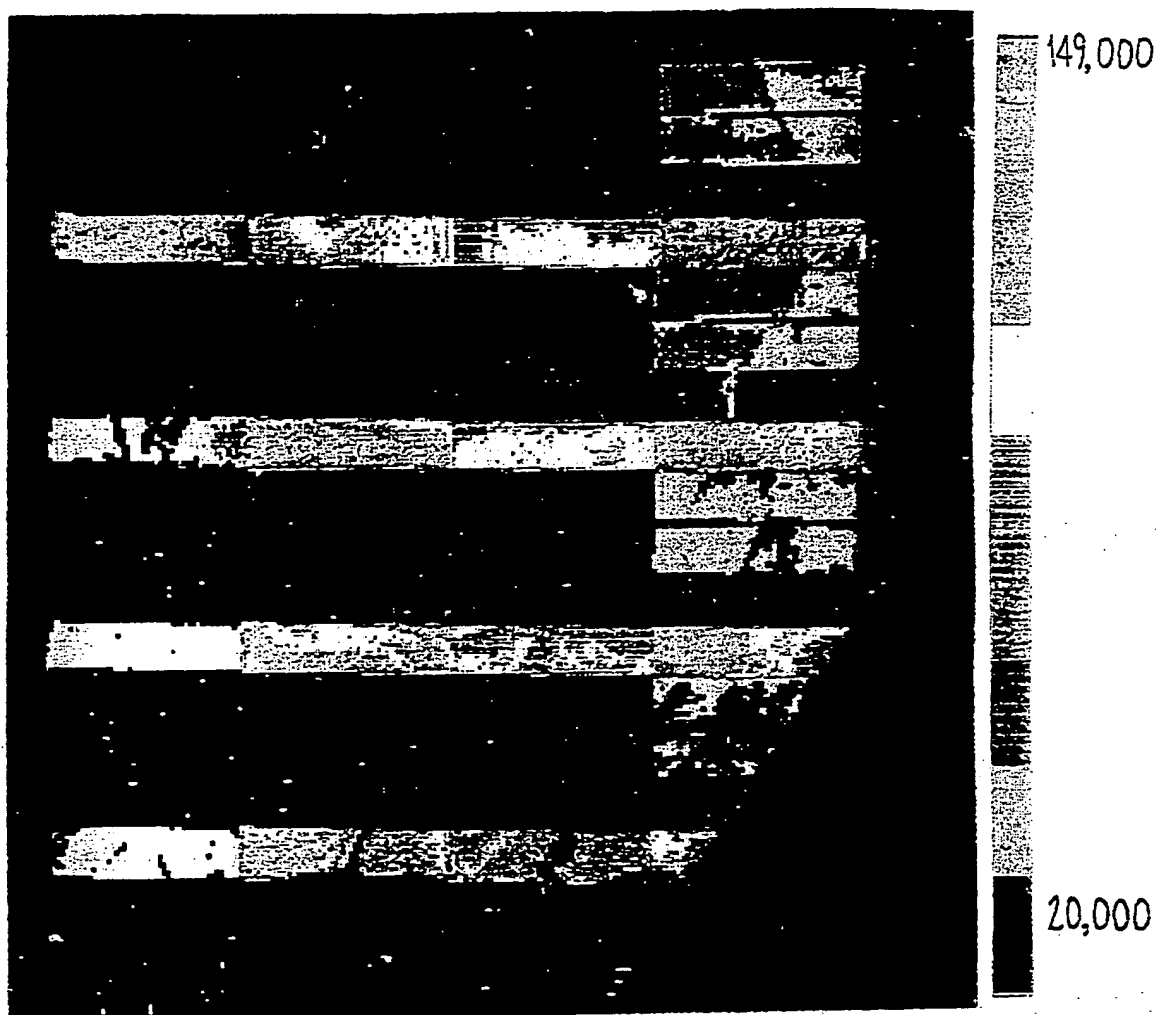


FIG. 19.

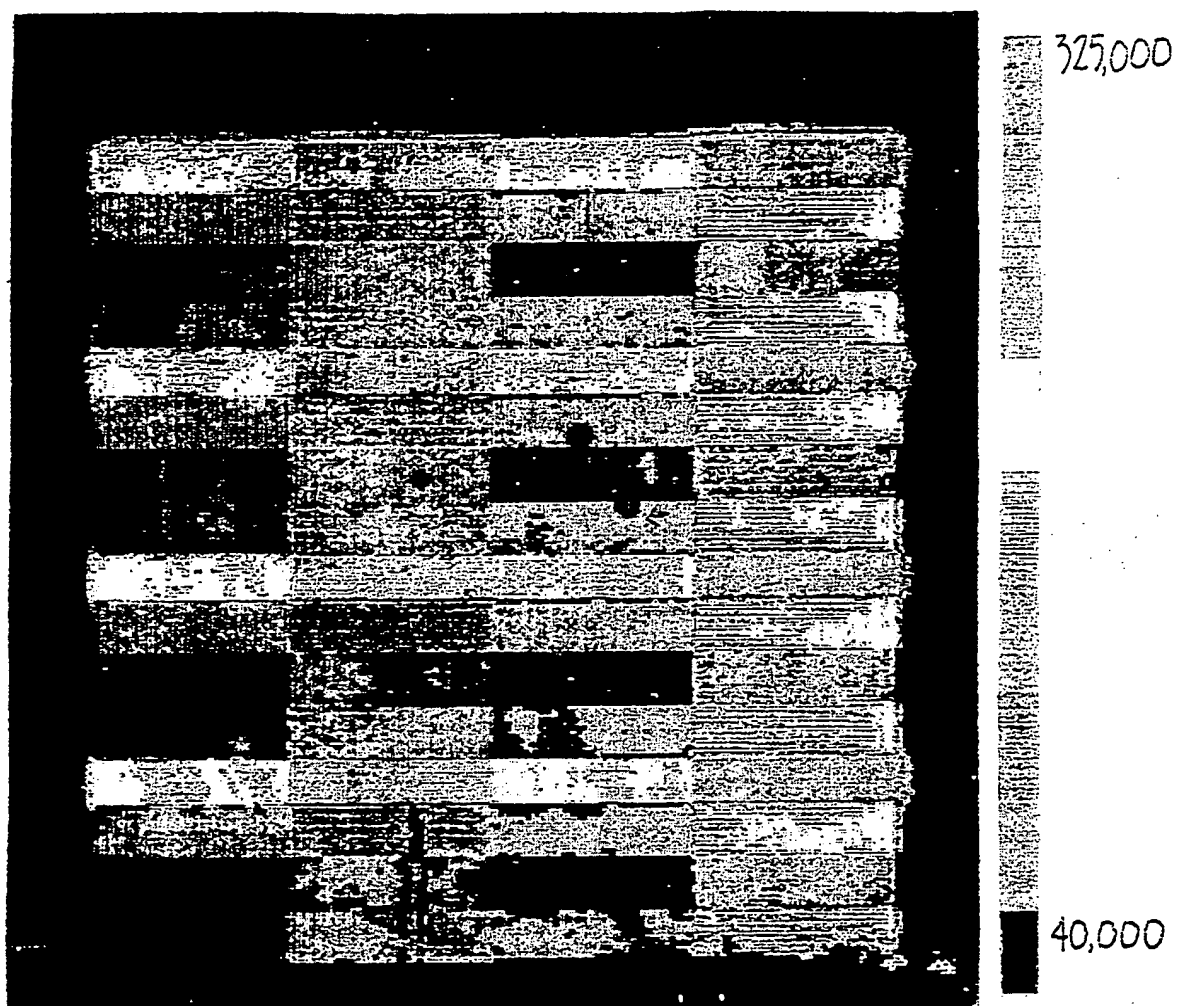


FIG. 20.

ARRAY OF OLIGONUCLEOTIDES ON A SOLID SUBSTRATE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a Rule 60 Division of U.S. application Ser. No. 850,356, filed Mar. 12, 1992, which is a Rule 60 Division of U.S. application Ser. No. 492,462, filed Mar. 7, 1990, now U.S. Pat. No. 5,143,854, which is a Continuation-in-Part of U.S. application Ser. No. 362,901, filed Jun. 7, 1989, now abandoned, all assigned to the assignee of the present invention.

The file of this patent contains drawings executed in color. Copies of this patent with color drawings will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

COPYRIGHT NOTICE

A portion of the disclosure of this patent document contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the Patent and Trademark Office patent file or records, but otherwise reserves all copyright rights whatsoever.

BACKGROUND OF THE INVENTION

The present inventions relate to the synthesis and placement of materials at known locations. In particular, one embodiment of the inventions provides a method and associated apparatus for preparing diverse chemical sequences at known locations on a single substrate surface. The inventions may be applied, for example, in the field of preparation of oligomer, peptide, nucleic acid, oligosaccharide, phospholipid, polymer, or drug congener preparation, especially to create sources of chemical diversity for use in screening for biological activity.

The relationship between structure and activity of molecules is a fundamental issue in the study of biological systems. Structure-activity relationships are important in understanding, for example, the function of enzymes, the ways in which cells communicate with each other, as well as cellular control and feedback systems.

Certain macromolecules are known to interact and bind to other molecules having a very specific three-dimensional spatial and electronic distribution. Any large molecule having such specificity can be considered a receptor, whether it is an enzyme catalyzing hydrolysis of a metabolic intermediate, a cell-surface protein mediating membrane transport of ions, a glycoprotein serving to identify a particular cell to its neighbors, an IgG-class antibody circulating in the plasma, an oligonucleotide sequence of DNA in the nucleus, or the like. The various molecules which receptors selectively bind are known as ligands.

Many assays are available for measuring the binding affinity of known receptors and ligands, but the information which can be gained from such experiments is often limited by the number and type of ligands which are available. Novel ligands are sometimes discovered by chance or by application of new techniques for the elucidation of molecular structure, including x-ray crystallographic analysis and recombinant genetic techniques for proteins.

Small peptides are an exemplary system for exploring the relationship between structure and function in biol-

ogy. A peptide is a sequence of amino acids. When the twenty naturally occurring amino acids are condensed into polymeric molecules they form a wide variety of three-dimensional configurations, each resulting from a particular amino acid sequence and solvent condition. The number of possible pentapeptides of the 20 naturally occurring amino acids, for example, is 20^5 or 3.2 million different peptides. The likelihood that molecules of this size might be useful in receptor-binding studies is supported by epitope analysis studies showing that some antibodies recognize sequences as short as a few amino acids with high specificity. Furthermore, the average molecular weight of amino acids puts small peptides in the size range of many currently useful pharmaceutical products.

Pharmaceutical drug discovery is one type of research which relies on such a study of structure-activity relationships. In most cases, contemporary pharmaceutical research can be described as the process of discovering novel ligands with desirable patterns of specificity for biologically important receptors. Another example is research to discover new compounds for use in agriculture, such as pesticides and herbicides.

Sometimes, the solution to a rational process of designing ligands is difficult or unyielding. Prior methods of preparing large numbers of different polymers have been painstakingly slow when used at a scale sufficient to permit effective rational or random screening. For example, the "Merrifield" method (*J. Am. Chem. Soc.* (1963) 85:2149-2154, which is incorporated herein by reference for all purposes) has been used to synthesize peptides on a solid support. In the Merrifield method, an amino acid is covalently bonded to a support made of an insoluble polymer. Another amino acid with an alpha protected group is reacted with the covalently bonded amino acid to form a dipeptide. After washing, the protective group is removed and a third amino acid with an alpha protective group is added to the dipeptide. This process is continued until a peptide of a desired length and sequence is obtained. Using the Merrifield method, it is not economically practical to synthesize more than a handful of peptide sequences in a day.

To synthesize larger numbers of polymer sequences, it has also been proposed to use a series of reaction vessels for polymer synthesis. For example, a tubular reactor system may be used to synthesize a linear polymer on a solid phase support by automated sequential addition of reagents. This method still does not enable the synthesis of a sufficiently large number of polymer sequences for effective economical screening.

Methods of preparing a plurality of polymer sequences are also known in which a porous container encloses a known quantity of reactive particles, the particles being larger in size than pores of the container. The containers may be selectively reacted with desired materials to synthesize desired sequences of product molecules. As with other methods known in the art, this method cannot practically be used to synthesize a sufficient variety of polypeptides for effective screening.

Other techniques have also been described. These methods include the synthesis of peptides on 96 plastic pins which fit the format of standard microtiter plates. Unfortunately, while these techniques have been somewhat useful, substantial problems remain. For example, these methods continue to be limited in the diversity of sequences which can be economically synthesized and screened.

From the above, it is seen that an improved method and apparatus for synthesizing a variety of chemical sequences at known locations is desired.

SUMMARY OF THE INVENTION

An improved method and apparatus for the preparation of a variety of polymers is disclosed.

In one preferred embodiment, linker molecules are provided on a substrate. A terminal end of the linker molecules is provided with a reactive functional group protected with a photoremovable protective group. Using lithographic methods, the photoremovable protective group is exposed to light and removed from the linker molecules in first selected regions. The substrate is then washed or otherwise contacted with a first monomer that reacts with exposed functional groups on the linker molecules. In a preferred embodiment, the monomer is an amino acid containing a photoremovable protective group at its amino or carboxy terminus and the linker molecule terminates in an amino or carboxy acid group bearing a photoremovable protective group.

A second set of selected regions is, thereafter, exposed to light and the photoremovable protective group on the linker molecule/protected amino acid is removed at the second set of regions. The substrate is then contacted with a second monomer containing a photoremovable protective group for reaction with exposed functional groups. This process is repeated to selectively apply monomers until polymers of a desired length and desired chemical sequence are obtained. Photolabile groups are then optionally removed and the sequence is, thereafter, optionally capped. Side chain protective groups, if present, are also removed.

By using the lithographic techniques disclosed herein, it is possible to direct light to relatively small and precisely known locations on the substrate. It is, therefore, possible to synthesize polymers of a known chemical sequence at known locations on the substrate.

The resulting substrate will have a variety of uses including, for example, screening large numbers of polymers for biological activity. To screen for biological activity, the substrate is exposed to one or more receptors such as antibodies whole cells, receptors on vesicles, lipids, or any one of a variety of other receptors. The receptors are preferably labeled with, for example, a fluorescent marker, radioactive marker, or a labeled antibody reactive with the receptor. The location of the marker on the substrate is detected with, for example, photon detection or autoradiographic techniques. Through knowledge of the sequence of the material at the location where binding is detected, it is possible to quickly determine which sequence binds with the receptor and, therefore, the technique can be used to screen large numbers of peptides. Other possible applications of the inventions herein include diagnostics in which various antibodies for particular receptors would be placed on a substrate and, for example, blood sera would be screened for immune deficiencies. Still further applications include, for example, selective "doping" of organic materials in semiconductor devices, and the like.

In connection with one aspect of the invention an improved reactor system for synthesizing polymers is also disclosed. The reactor system includes a substrate mount which engages a substrate around a periphery thereof. The substrate mount provides for a reactor space between the substrate and the mount through or into which reaction fluids are pumped or flowed. A

mask is placed on or focused on the substrate and illuminated so as to deprotect selected regions of the substrate in the reactor space. A monomer is pumped through the reactor space or otherwise contacted with the substrate and reacts with the deprotected regions. By selectively deprotecting regions on the substrate and flowing predetermined monomers through the reactor space, desired polymers at known locations may be synthesized.

Improved detection apparatus and methods are also disclosed. The detection method and apparatus utilize a substrate having a large variety of polymer sequences at known locations on a surface thereof. The substrate is exposed to a fluorescently labeled receptor which binds to one or more of the polymer sequences. The substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes a monochromatic or polychromatic light source for directing light at the substrate, means for detecting fluoresced light from the substrate, and means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the slide and data collection are recorded and managed by an appropriately programmed digital computer.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates masking and irradiation of a substrate at a first location. The substrate is shown in cross-section;

FIG. 2 illustrates the substrate after application of a monomer "A";

FIG. 3 illustrates irradiation of the substrate at a second location;

FIG. 4 illustrates the substrate after application of monomer "B";

FIG. 5 illustrates irradiation of the "A" monomer;

FIG. 6 illustrates the substrate after a second application of "B";

FIG. 7 illustrates a completed substrate;

FIGS. 8A and 8B illustrate alternative embodiments of a reactor system for forming a plurality of polymers on a substrate;

FIG. 9 illustrates a detection apparatus for locating fluorescent markers on the substrate;

FIGS. 10A-10M illustrate the method as it is applied to the production of the trimers of monomers "A" and "B";

FIGS. 11A and 11B are fluorescence traces for standard fluorescent beads;

FIGS. 12A and 12B are fluorescence curves for NVOC (6-nitroveratryloxycarbonyl) slides not exposed and exposed to light respectively;

FIGS. 13A to 13D are fluorescence plots of slides exposed through 100 μ m, 50 μ m, 20 μ m, and 10 μ m masks; 14A and 14B illustrate formation of YGGFL (a peptide of sequence H₂N-tyrosine-glycine-glycine-phenylalanine-leucine-CO₂H) and GGFL (a peptide of sequence H₂N-glycine-glycine-phenylalanine-leucine-CO₂H), followed by exposure to labeled Herz antibody (an antibody that recognizes YGGFL but not GGFL);

FIGS. 15A and 15B fluorescence plots of a slide with a checkerboard pattern of YGGFL and GGFL exposed to labeled Herz antibody; FIG. 15A illustrates a 500×500 μm mask which has been focused on the substrate according to FIG. 8A while FIG. 15B illustrates a 50×50 μm mask placed in direct contact with the substrate in accord with FIG. 8B;

FIG. 16 is a fluorescence plot of YGGFL and PGGFL synthesized in a 50 μm checkerboard pattern;

FIG. 17 is a fluorescence plot of YPGGFL and YGGFL synthesized in a 50 μm checkerboard pattern;

FIGS. 18A and 18B illustrate the mapping of sixteen sequences synthesized on two different glass slides;

FIG. 19 is a fluorescence plot of the slide illustrated in FIG. 18A; and

FIG. 20 is a fluorescence plot of the slide illustrated in FIG. 10B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS

I. Glossary	
II. General	
III. Polymer Synthesis	
IV. Details of One Embodiment of a Reactor System	25
V. Details of One Embodiment of a Fluorescent Detection Device	
VI. Determination of Relative Binding Strength of Receptors	
VII. Examples	30
A. Slide Preparation	
B. Synthesis of Eight Trimers of "A" and "B"	
C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group	
D. Demonstration of Signal Capability	35
E. Determination of the Number of Molecules Per Unit Area	
F. Removal of NVOC and Attachment of a Fluorescent Marker	
G. Use of a Mask in Removal of NVOC	40
H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse	
I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody	
J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL	45
K. Monomer-by Monomer Synthesis of YGGFL and YPGGFL	
L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody	50
VIII. Illustrative Alternative Embodiment	
IX. Conclusion	

I. Glossary
The following terms are intended to have the following general meanings as they are used herein:

1. Complementary: Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.
2. Epitope: The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.
3. Ligand: A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not

restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs (e.g., opiates, etc), lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

4. Monomer: A member of the set of small molecules which can be joined together to form a polymer. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer.

5. Peptide: A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer. Peptides are more than two amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, *Biochemistry*, Third Ed., 1988, which is incorporated herein by reference for all purposes.

6. Radiation: Energy which may be selectively applied including energy having a wavelength of between 10⁻¹⁴ and 10⁴ meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultraviolet radiation, visible light, infrared radiation, microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.

7. Receptor: A molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or man-made molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

Other examples of receptors which can be investigated by this invention include but are not restricted to:

- a) Microorganism receptors: Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.

- b) Enzymes: For instance, the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters; determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.
- c) Antibodies: For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for autoimmune diseases (e.g., by blocking the binding of the "self" antibodies).
- d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.
- e) Catalytic Polypeptides: Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. application Ser. No. 404,920, which is incorporated herein by reference for all purposes.
- f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which bind to a receptor may lead to the development of drugs to control blood pressure.
- g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.
8. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.
9. Protective Group: A material which is bound to a monomer unit and which may be spatially removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protective groups with utility herein include Nitroveratryloxy carbonyl, Nitrobenzyloxy carbonyl, Dimethyl dimethoxybenzyloxy carbonyl, 5-Bromo-7-nitroindolyl,

o-Hydroxy- α -methyl cinnamoyl, and 2-Oxymethylene anthraquinone. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.

10. Predefined Region: A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."
11. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.

II. General

The present invention provides methods and apparatus for the preparation and use of a substrate having a plurality of polymer sequences in predefined regions. The invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α -, β -, or ω -amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides.

The prepared substrate may, for example, be used in screening a variety of polymers as ligands for binding with a receptor, although it will be apparent that the invention could be used for the synthesis of a receptor for binding with a ligand. The substrate disclosed herein will have a wide variety of other uses. Merely by way of example, the invention herein can be used in determining peptide and nucleic acid sequences which bind to proteins, finding sequence-specific binding drugs, identifying epitopes recognized by antibodies, and evaluation of a variety of drugs for clinical and diagnostic applications, as well as combinations of the above.

The invention preferably provides for the use of a substrate "S" with a surface. Linker molecules "L" are optionally provided on a surface of the substrate. The purpose of the linker molecules, in some embodiments, is to facilitate receptor recognition of the synthesized polymers.

Optionally, the linker molecules may be chemically protected for storage purposes. A chemical storage protective group such as t-BOC (t-butoxycarbonyl) may be used in some embodiments. Such chemical protective groups would be chemically removed upon exposure to, for example, acidic solution and would serve to protect the surface during storage and be removed prior to polymer preparation.

On the substrate or a distal end of the linker molecules, a functional group with a protective group P_0 is provided. The protective group P_0 may be removed upon exposure to radiation, electric fields, electric cur-

rents, or other activators to expose the functional group.

In a preferred embodiment, the radiation is ultraviolet (UV), infrared (IR), or visible light. As more fully described below, the protective group may alternatively be an electrochemically-sensitive group which may be removed in the presence of an electric field. In still further alternative embodiments, ion beams, electron beams, or the like may be used for deprotection.

In some embodiments, the exposed regions and, therefore, the area upon which each distinct polymer sequence is synthesized are smaller than about 1 cm² or less than 1 mm². In preferred embodiments the exposed area is less than about 10,000 μm² or, more preferably, less than 100 μm² and may, in some embodiments, encompass the binding site for as few as a single molecule. Within these regions, each polymer is preferably synthesized in a substantially pure form.

Concurrently or after exposure of a known region of the substrate to light, the surface is contacted with a first monomer unit M₁ which reacts with the functional group which has been exposed by the deprotection step. The first monomer includes a protective group P₁. P₁ may or may not be the same as P₀.

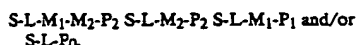
Accordingly, after a first cycle, known first regions of the surface may comprise the sequence:



while remaining regions of the surface comprise the sequence:

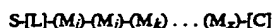


Thereafter, second regions of the surface (which may include the first region) are exposed to light and contacted with a second monomer M₂ (which may or may not be the same as M₁) having a protective group P₂. P₂ may or may not be the same as P₀ and P₁. After this second cycle, different regions of the substrate may comprise one or more of the following sequences:



The above process is repeated until the substrate includes desired polymers of desired lengths. By controlling the locations of the substrate exposed to light and the reagents exposed to the substrate following exposure, the location of each sequence will be known.

Thereafter, the protective groups are removed from some or all of the substrate and the sequences are, optionally, capped with a capping unit C. The process results in a substrate having a surface with a plurality of polymers of the following general formula:



where square brackets indicate optional groups, and M₁ ... M_n indicates any sequence of monomers. The number of monomers could cover a wide variety of values, but in a preferred embodiment they will range from 2 to 100.

In some embodiments a plurality of locations on the substrate polymers are to contain a common monomer subsequence. For example, it may be desired to synthesize a sequence S-M₁-M₂-M₃ at first locations and a sequence S-M₄-M₂-M₃ at second locations. The process would commence with irradiation of the first locations

followed by contacting with M₁-P, resulting in the sequence S-M₁-P at the first location. The second locations would then be irradiated and contacted with M₄-P, resulting in the sequence S-M₄-P at the second locations. Thereafter both the first and second locations would be irradiated and contacted with the dimer M₂-M₃, resulting in the sequence S-M₁-M₂-M₃ at the first locations and S-M₄-M₂-M₃ at the second locations. Of course, common subsequences of any length could be utilized including those in a range of 2 or more monomers, 2 to 100 monomers, 2 to 20 monomers, and a most preferred range of 2 to 3 monomers.

According to other embodiments, a set of masks is used for the first monomer layer and, thereafter, varied light wavelengths are used for selective deprotection. For example, in the process discussed above, first regions are first exposed through a mask and reacted with a first monomer having a first protective group P₁, which is removable upon exposure to a first wavelength of light (e.g., IR). Second regions are masked and reacted with a second monomer having a second protective group P₂, which is removable upon exposure to a second wavelength of light (e.g., UV). Thereafter, masks become unnecessary in the synthesis because the entire substrate may be exposed alternatively to the first and second wavelengths of light in the deprotection cycle.

The polymers prepared on a substrate according to the above methods will have a variety of uses including, for example, screening for biological activity. In such screening activities, the substrate containing the sequences is exposed to an unlabeled or labeled receptor such as an antibody, receptor on a cell, phospholipid vesicle, or any one of a variety of other receptors. In one preferred embodiment the polymers are exposed to a first, unlabeled receptor of interest and, thereafter, exposed to a labeled receptor-specific recognition element, which is, for example, an antibody. This process will provide signal amplification in the detection stage.

The receptor molecules may bind with one or more polymers on the substrate. The presence of the labeled receptor and, therefore, the presence of a sequence which binds with the receptor is detected in a preferred embodiment through the use of autoradiography, detection of fluorescence with a charge-coupled device, fluorescence microscopy, or the like. The sequence of the polymer at the locations where the receptor binding is detected may be used to determine all or part of a sequence which is complementary to the receptor.

Use of the invention herein is illustrated primarily with reference to screening for biological activity. The invention will, however, find many other uses. For example, the invention may be used in information storage (e.g., on optical disks), production of molecular electronic devices, production of stationary phases in separation sciences, production of dyes and brightening agents, photography, and in immobilization of cells, proteins, lectins, nucleic acids, polysaccharides and the like in patterns on a surface via molecular recognition of specific polymer sequences. By synthesizing the same compound in adjacent, progressively differing concentrations, a gradient will be established to control chemotaxis or to develop diagnostic dipsticks which, for example, titrate an antibody against an increasing amount of antigen. By synthesizing several catalyst molecules in close proximity, more efficient multistep conversions may be achieved by "coordinate immobilization." Co-

ordinate immobilization also may be used for electron transfer systems, as well as to provide both structural integrity and other desirable properties to materials such as lubrication, wetting, etc.

According to alternative embodiments, molecular biodistribution or pharmacokinetic properties may be examined. For example, to assess resistance to intestinal or serum proteases, polymers may be capped with a fluorescent tag and exposed to biological fluids of interest.

III. Polymer Synthesis

FIG. 1 illustrates one embodiment of the invention disclosed herein in which a substrate 2 is shown in cross-section. Essentially, any conceivable substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO₂, SiN₄, modified silicon, or any one of a wide variety of gels or polymers such as (poly)-tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface relief features of less than 10 Å.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si—OH functionalities, such as are found on silica surfaces.

The surface 4 of the substrate is preferably provided with a layer of linker molecules 6, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a

completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6–50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2–10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other linker molecules may be used in light of this disclosure.

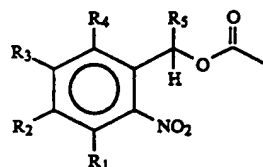
According to alternative embodiments, the linker molecules are selected based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to more closely approach the synthesized polymer.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or α,α-dimethyl-dimethoxybenzyloxycarbonyl (DDZ) is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:



where R₁ is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R₂ is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R₃ is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R₄ is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R₅ is alkyl, alkenyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include o-hydroxy- α -methyl cinnamoyl derivatives. Photoremovable protective groups are described in, for example, Patchornik, *J. Am. Chem. Soc.* (1970) 92:6333 and Amit et al., *J. Org. Chem.* (1974) 39:192, both of which are incorporated herein by reference.

In an alternative embodiment the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinnamate group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in light of this disclosure.

As shown in FIG. 1, the linking molecules are preferably exposed to, for example, light through a suitable mask 8 using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze, *VLSI Technology*, McGraw-Hill (1983), and Mead et al., *Introduction to VLSI Systems*, Addison-Wesley (1980), which are incorporated herein by reference for all purposes. The light may be directed at either the surface containing the protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups. In the embodiment shown in FIG. 1, light is directed at the surface of the substrate containing the protective groups. FIG. 1 illustrates the use of such masking techniques as they are applied to a positive reactive group so as to activate linking molecules and expose functional groups in areas 10a and 10b.

The mask 8 is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with, imaged on, or brought directly into contact with the substrate surface as shown in FIG. 1. "Openings" in the mask correspond to locations on the substrate where it is desired to remove photoremovable protective groups from the substrate. Alignment may be performed using conventional alignment techniques in which alignment marks (not shown) are used to accurately overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one described in Flanders et al., "A New Interferometric Alignment Technique," *App. Phys. Lett.* (1977) 31:426-428, which is incorporated herein by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may

comprise a molecule which is decomposed by light such as quinone diazide or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps. Several reactive groups along with their corresponding wavelengths for deprotection are provided in Table 1.

TABLE 1

Group	Approximate Deprotection Wavelength
Nitroveratryloxy carbonyl (NVOC)	UV (300-400 nm)
Nitrobenzyloxy carbonyl (NBOC)	UV (300-350 nm)
Dimethyl dimethoxybenzyloxy carbonyl	UV (280-300 nm)
5-Bromo-7-nitroindoliny	UV (420 nm)
o-Hydroxy- α -methyl cinnamoyl	UV (300-350 nm)
2-Oxymethylene anthraquinone	UV (350 nm)

While the invention is illustrated primarily herein by way of the use of a mask to illuminate selected regions of the substrate, other techniques may also be used. For example, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Pat. No. 4,719,615 (Feyrer et al.), which is incorporated herein by reference. In alternative embodiments a laser galvanometric scanner is utilized. In other embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled so as to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The substrate may be irradiated either in contact or not in contact with a solution (not shown) and is, preferably, irradiated in contact with a solution. The solution contains reagents to prevent the by-products formed by irradiation from interfering with synthesis of the polymer according to some embodiments. Such by-products might include, for example, carbon dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions. Alternatively, the solution may contain reagents used to match the index of refraction of the substrate. Reagents added to the solution may further include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH) or reagents known to react with a given functional group (e.g., aryl nitroso + glyoxylic acid \rightarrow aryl formhydroxamate + CO₂).

Either concurrently with or after the irradiation step, the linker molecules are washed or otherwise contacted with a first monomer, illustrated by "A" in regions 12a and 12b in FIG. 2. The first monomer reacts with the activated functional groups of the linkage molecules which have been exposed to light. The first monomer, which is preferably an amino acid, is also provided with a photoprotective group. The photoprotective group on the monomer may be the same as or different than the protective group used in the linkage molecules, and may be selected from any of the above-described protective groups. In one embodiment, the protective groups for the A monomer is selected from the group NBOC and NVOC.

As shown in FIG. 3, the process of irradiating is thereafter repeated, with a mask repositioned so as to remove linkage protective groups and expose functional groups in regions 14a and 14b which are illustrated as being regions which were protected in the previous masking step. As an alternative to repositioning of the first mask, in many embodiments a second mask will be utilized. In other alternative embodiments, some steps may provide for illuminating a common region in successive steps. As shown in FIG. 3, it may be desirable to provide separation between irradiated regions. For example, separation of about 1-5 μm may be appropriate to account for alignment tolerances.

As shown in FIG. 4, the substrate is then exposed to a second protected monomer "B," producing B regions 16a and 16b. Thereafter, the substrate is again masked so as to remove the protective groups and expose reactive groups on A region 12a and B region 16b. The substrate is again exposed to monomer B, resulting in the formation of the structure shown in FIG. 6. The dimers B-A and B-B have been produced on the substrate.

A subsequent series of masking and contacting steps similar to those described above with A (not shown) provides the structure shown in FIG. 7. The process provides all possible dimers of B and A, i.e., B-A, A-B, A-A, and B-B.

The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes, may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment the regions 12a, 12b and 16a, 16b on the substrate will have a surface area of between about 1 cm^2 and 10^{-10} cm^2 . In some embodiments the regions 12a, 12b and 16a, 16b have areas of less than about 10^{-1} cm^2 , 10^{-2} cm^2 , 10^{-3} cm^2 , 10^{-4} cm^2 , 10^{-5} cm^2 , 10^{-6} cm^2 , 10^{-7} cm^2 , 10^{-8} cm^2 , or 10^{-10} cm^2 . In a preferred embodiment, the regions 12a, 12b and 16a, 16b are between about $10 \times 10 \mu\text{m}$ and $500 \times 500 \mu\text{m}$.

In some embodiments a single substrate supports more than about 10 different monomer sequences and preferably more than about 100 different monomer sequences, although in some embodiments more than about 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , or 10^8 different sequences are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% pure.

According to some embodiments, several sequences are intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated.

IV. Details of One Embodiment of a Reactor System

FIG. 8A schematically illustrates a preferred embodiment of a reactor system 100 for synthesizing polymers on the prepared substrate in accordance with one aspect of the invention. The reactor system includes a body 102 with a cavity 104 on a surface thereof. In preferred embodiments the cavity 104 is between about 50 and 1000 μm deep with a depth of about 500 μm preferred.

The bottom of the cavity is preferably provided with an array of ridges 106 which extend both into the plane of the Figure and parallel to the plane of the Figure. The ridges are preferably about 50 to 200 μm deep and spaced at about 2 to 3 mm. The purpose of the ridges is to generate turbulent flow for better mixing. The bottom surface of the cavity is preferably light absorbing so as to prevent reflection of impinging light.

A substrate 112 is mounted above the cavity 104. The substrate is provided along its bottom surface 114 with a photoremovable protective group such as NVOC with or without an intervening linker molecule. The substrate is preferably transparent to a wide spectrum of light, but in some embodiments is transparent only at a wavelength at which the protective group may be removed (such as UV in the case of NVOC). The substrate in some embodiments is a conventional microscope glass slide or cover slip. The substrate is preferably as thin as possible, while still providing adequate physical support. Preferably, the substrate is less than about 1 mm thick, more preferably less than 0.5 mm thick, more preferably less than 0.1 mm thick, and most preferably less than 0.05 mm thick. In alternative preferred embodiments, the substrate is quartz or silicon.

The substrate and the body serve to seal the cavity except for an inlet port 108 and an outlet port 110. The body and the substrate may be mated for sealing in some embodiments with one or more gaskets. According to a preferred embodiment, the body is provided with two concentric gaskets and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets. Fluid is pumped through the inlet port into the cavity by way of a pump 116 which may be, for example, a model no. B-120-S made by Eldex Laboratories. Selected fluids are circulated into the cavity by the pump, through the cavity, and out the outlet for recirculation or disposal. The reactor may be subjected to ultrasonic radiation and/or heated to aid in agitation in some embodiments.

Above the substrate 112, a lens 120 is provided which may be, for example, a 2"100 mm focal length fused silica lens. For the sake of a compact system, a reflective mirror 122 may be provided for directing light from a light source 124 onto the substrate. Light source 124 may be, for example, a Xe(Hg) light source manufactured by Oriel and having model no. 66024. A second lens 126 may be provided for the purpose of projecting a mask image onto the substrate in combination with lens 120. This form of lithography is referred to herein as projection printing. As will be apparent from this disclosure, proximity printing and the like may also be used according to some embodiments.

Light from the light source is permitted to reach only selected locations on the substrate as a result of mask 128. Mask 128 may be, for example, a glass slide having

etched chrome thereon. The mask 128 in one embodiment is provided with a grid of transparent locations and opaque locations. Such masks may be manufactured by, for example, Photo Sciences, Inc. Light passes freely through the transparent regions of the mask, but is reflected from or absorbed by other regions. Therefore, only selected regions of the substrate are exposed to light.

As discussed above, light valves (LCD's) may be used as an alternative to conventional masks to selectively expose regions of the substrate. Alternatively, fiber optic faceplates such as those available from Schott Glass, Inc, may be used for the purpose of contrast enhancement of the mask or as the sole means of restricting the region to which light is applied. Such faceplates would be placed directly above or on the substrate in the reactor shown in FIG. 8A. In still further embodiments, flys-eye lenses, tapered fiber optic faceplates, or the like, may be used for contrast enhancement.

In order to provide for illumination of regions smaller than a wavelength of light, more elaborate techniques may be utilized. For example, according to one preferred embodiment, light is directed at the substrate by way of molecular microcrystals on the tip of, for example, micropipettes. Such devices are disclosed in Lieberman et al., "A Light Source Smaller Than the Optical Wavelength," *Science* (1990) 247:59-61, which is incorporated herein by reference for all purposes.

In operation, the substrate is placed on the cavity and sealed thereto. All operations in the process of preparing the substrate are carried out in a room lit primarily or entirely by light of a wavelength outside of the light range at which the protective group is removed. For example, in the case of NVOC, the room should be lit with a conventional dark room light which provides little or no UV light. All operations are preferably conducted at about room temperature.

A first, deprotection fluid (without a monomer) is circulated through the cavity. The solution preferably is of 5 mM sulfuric acid in dioxane solution which serves to keep exposed amino groups protonated and decreases their reactivity with photolysis by-products. Absorptive materials such as N,N-diethylamino 2,4-dinitrobenzene, for example, may be included in the deprotection fluid which serves to absorb light and prevent reflection and unwanted photolysis.

The slide is, thereafter, positioned in a light raypath from the mask such that first locations on the substrate are illuminated and, therefore, deprotected. In preferred embodiments the substrate is illuminated for between about 1 and 15 minutes with a preferred illumination time of about 10 minutes at 10-20 mW/cm² with 365 nm light. The slides are neutralized (i.e., brought to a pH of about 7) after photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated in bulk, and then reinstalled in the flow cell. Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump 116. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its α -nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container 118,

through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2 provides an illustration of a mixture which may be used for solution A.

TABLE 2

Representative Monomer Carrier Solution "A"
100 mg NVOC amino protected amino acid
37 mg HOBT (1-Hydroxybenzotriazole)
250 μ l DMF (Dimethylformamide)
86 μ l DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500 μ l are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

TABLE 3

Representative Monomer Carrier Solution "B"
250 μ l DMF
111 mg BOP (Benzotriazolyl-n-oxy-tris(dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of removing the slide from the reactor and submersing it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50 times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light 124 is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example, a solution of about 1% BSA (bovine serum albumin), 0.5% Tween TM non-ionic detergent in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4 μ g/ml.

FIG. 8B illustrates an alternative preferred embodiment of the reactor shown in FIG. 8A. According to

this embodiment, the mask 128 is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the imaging lenses 120 and 126 are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

If, for example, it is desired to synthesize all 16 dinu-

pled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

TABLE 4

Mask Strategy Program

```

DEFINT A-Z
DIM b(20), w(20), l(500)
FS = "LPT1:"
OPEN FS FOR OUTPUT AS #1
jmax = 3      'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5      'Number of building blocks for res 1,2,3
g = 1: lmax(1) = 1
FOR j = 1 TO jmax: g = g * b(j): NEXT j
w(0) = 0: w(1) = g / b(1)
PRINT #1, "MASK2.BAS", DATE$, TIME$: PRINT #1,
PRINT #1, USING "Number of residues=###": jmax
FOR j = 1 TO jmax
PRINT #1, USING "    Residue ##    ## building blocks": j; b(j)
NEXT j
PRINT #1, "
PRINT #1, USING "Number of cells=#####": g: PRINT #1,
FOR j = 2 TO jmax
lmax(j) = lmax(j-1) * b(j-1)
w(j) = w(j-1) / b(j)
NEXT j
FOR j = 1 TO jmax
PRINT #1, USING "Mask for residue ##": j: PRINT #1,
PRINT #1, USING "    Number of stripes=#####": lmax(j)
PRINT #1, USING "    Width of each stripe=#####": w(j)
FOR l = 1 TO lmax(j)
a = l + (1 - 1) * w(j - 1)
ac = a + w(j) - 1
PRINT #1, USING "    Stripe ## begins at location ### and ends at ###": l; a; ac
NEXT l
PRINT #1,
PRINT #1, USING "    For each of ## building blocks, translate mask by ##
cell(s)": b(j); w(j);
PRINT #1, : PRINT #1, : PRINT #1,
NEXT j

```

© Copyright 1990, Affymax Research Institute

cleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is cou-

TABLE 5

Masking Strategy Output

Number of residues=	3
Residue 1	3 building blocks
Residue 2	4 building blocks
Residue 3	5 building blocks

TABLE 5-continued

Masking Strategy Output	
Number of cells= 60	
Mask for residue 1	
Number of stripes= 1	
Width of each stripe= 20	
Stripe 1 begins at location 1 and ends at 20	
For each of 3 building blocks, translate mask by 20 cell(s)	
Mask for residue 2	
Number of stripes= 3	
Width of each stripe= 5	
Stripe 1 begins at location 1 and ends at 5	
Stripe 2 begins at location 21 and ends at 25	
Stripe 3 begins at location 41 and ends at 45	
For each of 4 building blocks, translate mask by 5 cell(s)	
Mask for residue 3	
Number of stripes= 12	
Width of each stripe= 1	
Stripe 1 begins at location 1 and ends at 1	
Stripe 2 begins at location 6 and ends at 6	
Stripe 3 begins at location 11 and ends at 11	
Stripe 4 begins at location 16 and ends at 16	
Stripe 5 begins at location 21 and ends at 21	
Stripe 6 begins at location 26 and ends at 26	
Stripe 7 begins at location 31 and ends at 31	
Stripe 8 begins at location 36 and ends at 36	
Stripe 9 begins at location 41 and ends at 41	
Stripe 10 begins at location 46 and ends at 46	
Stripe 11 begins at location 51 and ends at 51	
Stripe 12 begins at location 56 and ends at 56	
For each of 5 building blocks, translate mask by 1 cell(s)	

© Copyright 1990, Affymax Research Institute

V. Details of One Embodiment of A Fluorescent Detection Device

FIG. 9 illustrates a fluorescent detection device for detecting fluorescently labeled receptors on a substrate. A substrate 112 is placed on an x/y translation table 202. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer 204 which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope 206 which includes one or more objectives 208. Light (about 488 nm) from a laser 210, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror 207 which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror 207 may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope 206 which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter 209 and, thereafter through an aperture plate 211. Wavelength filter 209 may be, for example, a model no. OG530 manufactured by Melles Griot and

aperture plate 211 may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube 212 which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter 216. The number of photons is recorded as a function of the location in the computer 204. Pre-Amp 214 may be, for example, a model no. SR440 manufactured by Stanford Research Systems and photon counter 216 may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100 μm with a data collection diameter of about 0.8 to 10 μm preferred. In embodiments with sufficiently high fluorescence, a CCD (charge coupled device) detector with broadfield illumination is utilized.

By counting the number of photons generated in a given area in response to the laser, it is possible to determine where fluorescent marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescently marked receptor.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

VI. Determination of Relative Binding Strength of Receptors

The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

VII. Examples

The following examples are provided to illustrate the efficacy of the inventions herein. All operations were

conducted at about ambient temperatures and pressures unless indicated to the contrary.

A. Slide Preparation

Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip. According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from 10⁻⁷% to 10% may be used, with about 10⁻³% to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters (μ l) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500 μ l of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110°-120° C. vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500 μ l of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface—that is, those amino groups which have not had the NVOC-GABA attached—are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

B. Synthesis of Eight Trimers of "A" and "B"

FIG. 10 illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitroveratryloxycarbonyl (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photoreactive at the wavelength of light used to protect the primary chain.

For a monomer set of size n , $n \times l$ cycles are required to synthesize all possible sequences of length l . A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue

is to be added, with appropriate washes to remove the by-products of the deprotection.

2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in FIG. 10A, the glass is provided with regions 22, 24, 26, 28, 30, 32, 34, and 36. Regions 30, 32, 34, and 36 are masked, as shown in FIG. 10B and the glass is irradiated and exposed to a reagent containing "A" (e.g., gly), with the resulting structure shown in FIG. 10C. Thereafter, regions 22, 24, 26, and 28 are masked, the glass is irradiated (as shown in FIG. 10D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in FIG. 10E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in FIG. 10M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

$$n \times l \quad (1)$$

where:

n = the number of monomers in the basis set of monomers, and

l = the number of monomer units in a polymer chain.

Conversely, the synthesized number of sequences of length l will be:

$$n^l \quad (2)$$

Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than l . If, in the extreme case, all polymers having a length less than or equal to l are synthesized, the number of polymers synthesized will be:

$$n^l + n^{l-1} + \dots + n^1 \quad (3)$$

The maximum number of lithographic steps needed will generally be n for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be $n \times l$. The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and

the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas} = (A) / (\text{Sequences})$$

where:

A is the total area available for synthesis; and
Sequences is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as a substrate. The durapore membrane was a polyvinylidene difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between -70° and $+50^{\circ}$ C.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standards and having model no. 824. This kit includes $5.8 \mu\text{m}$ diameter beads, each im-

pregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in FIG. 9 in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000 and 13,000; fluorescein molecules, are shown in FIGS. 11A and 11B respectively. On each curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100 μW of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of photons per fluorescein molecule that can be detected. For the curves illustrated in FIG. 11A and 11B, this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40-50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then dried and stored in the dark until it was ready to be examined.

Through the use of curves similar to those shown in FIG. 11A and 11B, and by integrating the fluorescent counts under the exponentially decaying signal, the number of free amino groups on the surface after derivatization was determined. It was determined that slides with labeling densities of 1 fluorescein per $10^3 \times 10^3$ to $\sim 2 \times 2 \text{ nm}$ could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from 10-5% to 10-1%.

F. Removal of NVOC and Attachment of A Fluorescent Marker

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

FIG. 12A illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm², ~350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in FIG. 12B. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

G. Use of a Mask in Removal of NVOC

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg—Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position color-coded for fluorescence intensity. The fluorescence intensity (in counts) as a function of location is given on the color scale to the right of FIG. 13A for a mask having 100×100 μm squares.

The experiment was repeated a number of times through various masks. The fluorescence pattern for a 50 μm mask is illustrated in FIG. 13B, for a 20 μm mask in FIG. 13C, and for a 10 μm mask in FIG. 13D. The mask pattern is distinct down to at least about 10 μm squares using this lithographic technique.

H. Attachment of YGGFL and Subsequent Exposure to

Herz Antibody and Goat Antimouse

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500 μm checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H₂N-tyrosine, glycine, glycine, phenylalanine, leucine-CO₂H, otherwise referred to herein as YGGFL) was attached via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBT/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes at 2 μg/ml in a supercocktail (containing 1% BSA and 1% ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2 μg/ml in the supercocktail buffer, and allowed to incubate for 2 hours. An image taken at 10 μm steps indicated that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provides for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide is available for binding with an antibody, and (3) that the detection apparatus capabilities are sufficient to detect binding of a receptor.

I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment and the results thereof are illustrated in FIGS. 14A, 14B, 15A, and 15B.

In FIG. 14A, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated and coupled to NVOC-glycine to form the sequence shown in the last portion of FIG. 14A.

As shown in FIG. 14B, alternating regions of the slide were then illuminated using a projection print using a 500×500 μm checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan is shown in FIG. 15A, and the color coding for the fluorescence intensity is again given on the right. Dark areas contain the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluorescein conjugate), and in the red areas YGGFL is present. The YGGFL pentapeptide is recognized by the Herz antibody and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns are shown for a 50 μm mask used in direct contact ("proximity print") with the substrate in FIG. 15B. Note that the pattern is more distinct and the corners of the checkerboard pattern are touching when the mask is placed in direct contact with the substrate (which reflects the increase in resolution using this technique).

J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL

A synthesis using a 50 μm checkerboard mask similar to that shown in FIG. 15B was conducted. However, P was added to the GGFL sites on the substrate through an additional coupling step. P was added by exposing protected GGFL to light and subsequent exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment is provided in FIG. 16. As shown, the regions are again readily discernable. This experiment demonstrates that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

K. Monomer-by-Monomer Synthesis of YGGFL and YPGGFL

In order to further demonstrate the operability of the invention, a 50 μ m checkerboard pattern of alternating YGGFL and YPGGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot is provided in FIG. 17. Again, it is seen that the antibody is clearly able to recognize the YGGFL sequence and does not bind significantly at the YPGGFL regions.

L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm \times 0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. FIGS. 18A and 18B illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in FIGS. 18A and 18B were duplicated four times on each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

FIG. 19 is a fluorescence plot of the first slide, which contained only L amino acids. Red indicates strong binding (149,000 counts or more) while black indicates little or no binding of the Herz antibody (20,000 counts or less). The bottom right-hand portion of the slide appears "cut off" because the slide was broken during processing. The sequence YGGFL is clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibit strong recognition of the antibody. By contrast, most of the remaining sequences show little or no binding. The four duplicate portions of the slide are extremely consistent in the amount of binding shown therein.

FIG. 20 is a fluorescence plot of the second slide. Again, strongest binding is exhibited by the YGGFL sequence. Significant binding is also detected to YaGFL, YsGFL, and YpGFL (where L-amino acids are identified by one upper case letter abbreviation, and D-amino acids are identified by one lower case letter abbreviation). The remaining sequences show less binding with the antibody. Note the low binding efficiency of the sequence yGGFL.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

TABLE 6

Apparent Binding to Herz Ab	
L-aa Set	D-aa Set
YGGFL	YGGFL
YAGFL	YaGFL
YSGFL	YsGFL
LGGFL	YpGFL
FGGFL	fGGFL
YPGFL	yGGFL
LAGFL	faGFL
FAGFL	wGGFL
WGGFL	yaGFL
	fpGFL

TABLE 6-continued

Apparent Binding to Herz Ab	
L-aa Set	D-aa Set
	waGFL

VIII. Illustrative Alternative Embodiment

According to an alternative embodiment of the invention, the methods provide for attaching to the surface a caged binding member which in its caged form has a relatively low affinity for other potentially binding species, such as receptors and specific binding substances.

According to this alternative embodiment, the invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing receptors. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined regions. The caged binding members are liberated to act as binding members ultimately capable of binding receptors upon selective activation of the predefined regions. The activated binding members are then used to immobilize specific molecules such as receptors on the predefined region of the surface. The above procedure is repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing, for example, the same or different receptors. When receptors immobilized in this way have a differential affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the receptors.

The alternative embodiment may make use of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low affinity for receptors of substances that specifically bind to uncaged binding members when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected from reaction until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups labilize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to a receptor. The receptor chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The receptor will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the receptor retains its activity toward a particular ligand.

Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin complex, i.e., a biotin molecule that has been chemically modified with photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs than does natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give

binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog is incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface can then be incubated with a desired receptor or conjugate of a desired receptor. The receptor will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated receptor complex, which has been previously prepared, to activated binding members on the surface.

IX. Conclusion

The present inventions provide greatly improved methods and apparatus for synthesis of polymers on substrates. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with reference to the use of photoremovable protective groups, but it will be readily recognized by those of skill in the art that sources of radiation other than light could also be used. For example, in some embodiments it may be desirable to use protective groups which are sensitive to electron beam irradiation, x-ray irradiation, in combination with electron beam lithograph, or x-ray lithography techniques. Alternatively, the group could be removed by exposure to an electric current. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A substrate with a surface comprising 10^3 or more groups of oligonucleotides with different, known sequences covalently attached to the surface in discrete known regions, said 10^3 or more groups of oligonucleotides occupying a total area of less than 1 cm^2 on said substrate, said groups of oligonucleotides having different nucleotide sequences.

2. The substrate as recited in claim 1 wherein said substrate comprises 10^4 or more different groups of oligonucleotide with known sequences covalently coupled to discrete known regions of said substrate.

3. The substrate as recited in claim 1 wherein said substrate comprises 10^5 or more different groups of oligonucleotides with known sequences in discrete known regions.

4. The substrate as recited in claim 1 wherein said substrate comprises 10^6 or more different groups of oligonucleotides with known sequences in discrete known regions.

5. The substrate as recited in claim 1 wherein said groups of oligonucleotides are at least 50% pure within said discrete known regions.

6. The substrate as recited in claim 1 wherein the groups of oligonucleotides are attached to the surface by a linker.

7. An array of more than 1,000 different groups of oligonucleotide molecules with known sequences covalently coupled to a surface of a substrate, said groups of oligonucleotide molecules each in discrete known regions and differing from other groups of oligonucleotide molecules in monomer sequence, each of said discrete known regions being an area of less than about 0.01 cm^2 and each discrete known region comprising oligonucleotides of known sequence, said different groups occupying a total area of less than 1 cm^2 .

8. The array as recited in claim 7 wherein said area is less than $10,000 \text{ microns}^2$.

9. The array as recited in claim 7 made by the process of:

exposing a first region of said substrate to light to remove photoremovable groups from nucleic acids in said first region, and not exposing a second region of said surface to light;

covalently coupling a first nucleotide to said nucleic acids on said part of said substrate exposed to light, said first nucleotide covalently coupled to said photoremovable group;

exposing a part of said first region of said substrate to light, and not exposing another part of said first region of said substrate to light to remove said photoremovable groups;

covalently coupling a second nucleotide to said part of said first region exposed to light; and

repeating said steps of exposing said substrate to light and covalently coupling nucleotides until said more than 500 different groups of nucleotides are formed on said surface.

10. The array as recited in claim 7 comprising more than 10,000 groups of oligonucleotides of known sequences.

* * * * *

F

This Page Blank (uspto)

United States Patent [19]

Lockhart et al.

[11] Patent Number: 5,556,752

[45] Date of Patent: Sep. 17, 1996

[54] SURFACE-BOUND, UNIMOLECULAR, DOUBLE-STRANDED DNA

[75] Inventors: David J. Lockhart, Santa Clara, Calif.;
Dirk Vetter, Freiburg, Germany;
Martin Diggelmann, Niederdorf,
Switzerland

[73] Assignee: Affymetrix, Inc., Santa Clara, Calif.

[21] Appl. No.: 327,687

[22] Filed: Oct. 24, 1994

[51] Int. Cl.⁶ C12Q 1/68; C07H 21/00

[52] U.S. Cl. 435/6; 536/23.1

[58] Field of Search 435/6; 536/23.1;
530/413

[56] References Cited

U.S. PATENT DOCUMENTS

4,376,110	3/1983	David et al.	435/5
4,562,157	12/1985	Lowe et al.	435/287.2
4,728,502	3/1988	Hamill	422/116
5,143,834	9/1992	Pirung et al.	436/518
5,288,514	2/1994	Ellman	165/155

FOREIGN PATENT DOCUMENTS

WO89/10977	11/1989	WIPO
WO89/11548	11/1989	WIPO
WO90/00626	1/1990	WIPO
WO90/15070	12/1990	WIPO
WO92/00091	1/1992	WIPO

OTHER PUBLICATIONS

Duncan, C. H. et al (1988) Analytical Biochemistry 169: 104-108. "Affinity Chromatography of a Sequence-specific DNA binding protein using Teflon linked . . .".
Ma, M. Y.-X. et al (1993) Biochemistry 32: 1751-1758. "Design & Synthesis of RNA Miniduplicates via a synthetic linker approach." Markiewicz, W T et al (1989) Nucleic Acids Research 17: 7149-7157. "Universal solid supports for the synthesis of oligonucleotides with 3'-PO₄s".

Ohlmeyer, M H J et al (1993) Proc. Natl. Acad. Sci. USA 90: 10922-10926 "Complex Synthetic Chemical Libraries Indexed with molecular Tags." Geysen, et al., *J. Immun. Meth.* 102:259-274 (1987).
Frank and Doring, *Tetrahedron*, 44:6031-6040 (1988).
Fodor et al., *Science*, 251:767-777 (1991).
Lam et al., *Nature*, 354:82-84 (1991).
Houghien et al., *Nature*, 354:84-86 (1991).
Galas et al., *Nucleic Acid Res.* 5(9):3157-3170 (1978).
Murphy et al., *Science* 262:1025-1029 (1993).
Lysov et al., *Dokl. Akad. Nauk SSSR*, 303:1508-1511 (1988) (See footnote provided, P. 436).
Bains et al., *J. Theor. Biol.*, 135:303-307 (1988).
Drmanac et al., *Genomics*, 4:114-128 (1989).
Sreznoska et al., *Proc. Natl. Acad. Sci. USA*, 88:10089-10093 (1991).
Drmanac et al., *Science*, 260:1649-1652 (1993).
Needels, et al., *Proc. Natl. Acad. Sci. USA*, 90:10700-10704 (1993).
Scaria, P. V., et al. *J. of Biol. Chem.*, 266(9): 5417-5423 (1993).

(List continued on next page.)

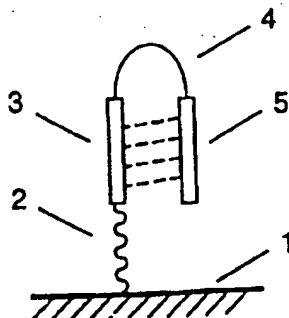
Primary Examiner—Mindy Fleisher
Assistant Examiner—Scott David Prieb
Attorney, Agent, or Firm—Townsend and Townsend and Crew LLP

[57]

ABSTRACT

Libraries of unimolecular, double-stranded oligonucleotides on a solid support. These libraries are useful in pharmaceutical discovery for the screening of numerous biological samples for specific interactions between the double-stranded oligonucleotides, and peptides, proteins, drugs and RNA. In a related aspect, the present invention provides libraries of conformationally restricted probes on a solid support. The probes are restricted in their movement and flexibility using double-stranded oligonucleotides as scaffolding. The probes are also useful in various screening procedures associated with drug discovery and diagnosis. The present invention further provides methods for the preparation and screening of the above libraries.

6 Claims, 1 Drawing Sheet



OTHER PUBLICATIONS

- Durand, M., et al., *Nucleic Acid Res.*, 18(21) : 6353-5469 (1990).
- Farnulok, M., et al., *Angew. Chem. Int. Ed. Engl.*, 31:979-988 (1992).
- Chanopadhyaya, R., et al., *Nature*, 334:175-179 (1988).
- Bock, L. C., et al., *Nature*, 355:564-566 (1992).
- Parham, Peter, *Nature*, 360:300-301 (1992).
- Tuerk, C., et al., *Science*, 249:505-510 (1990).
- Mergny, J.-L., et al., *Nucleic Acids Res.*, 19(7) : 1521-1526 (1991).
- Brossalina, E., et al., *J. Am. Chem. Soc.*, 115:796-797 (1993).
- Härd, T., et al., *Biochemistry*, 29:959-965 (1990).
- Cook, J., et al., *Analytical Biochemistry*, 190:331-339 (1990).
- Cuniberti, C. et al., *Biophysical Chemistry*, 38:11-22 (1990).
- Berman, H. M., et al., *Ann. Rev. Biophys. Bioeng.*, 10:87-114 (1981).
- White et al. "Principles of Biochemistry" New York: McGraw-Hill, 1978 pp. 124-128.

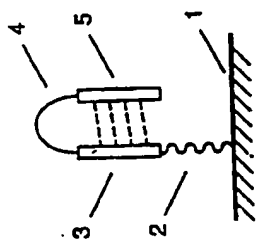


Fig. 1c

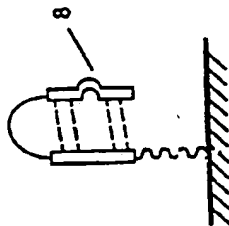


Fig. 1f

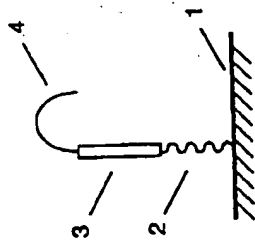


Fig. 1b

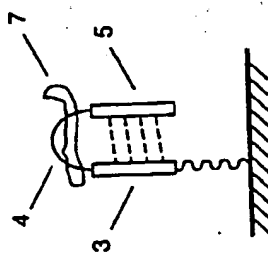


Fig. 1e

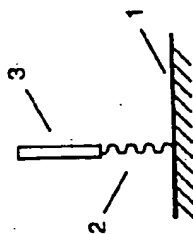


Fig. 1a

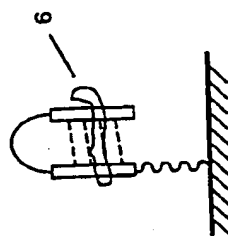


Fig. 1d

Figure 1a-1f

SURFACE-BOUND, UNIMOLECULAR, DOUBLE-STRANDED DNA

GOVERNMENT RIGHTS

Research leading to the invention was funded in part by NIH Grant No. R01HG00813-03 and the government may have certain rights to the invention.

BACKGROUND OF THE INVENTION

The present invention relates to the field of polymer synthesis and the use of polymer libraries for biological screening. More specifically, in one embodiment the invention provides arrays of diverse double-stranded oligonucleotide sequences. In another embodiment, the invention provides arrays of conformationally restricted probes, wherein the probes are held in position using double-stranded DNA sequences as scaffolding. Libraries of diverse unimolecular double-stranded nucleic acid sequences and probes may be used, for example, in screening studies for determination of binding affinity exhibited by binding proteins, drugs, or RNA.

Methods of synthesizing desired single stranded DNA sequences are well known to those of skill in the art. In particular, methods of synthesizing oligonucleotides are found in, for example, *Oligonucleotide Synthesis: A Practical Approach*, Gait, ed., IRL Press, Oxford (1984), incorporated herein by reference in its entirety for all purposes. Synthesizing unimolecular double-stranded DNA in solution has also been described. See, Durand, et al. *Nucleic Acids Res.* 18:6353-6359 (1990) and Thomson, et al. *Nucleic Acids Res.* 21:5600-5603 (1993), the disclosures of both being incorporated herein by reference.

Solid phase synthesis of biological polymers has been evolving since the early "Merrifield" solid phase peptide synthesis, described in Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963), incorporated herein by reference for all purposes. Solid-phase synthesis techniques have been provided for the synthesis of several peptide sequences on, for example, a number of "pins." See e.g., Geysen et al., *J. Immun. Meth.* 102:259-274 (1987), incorporated herein by reference for all purposes. Other solid-phase techniques involve, for example, synthesis of various peptide sequences on different cellulose disks supported in a column. See Frank and Doring, *Tetrahedron* 44:6031-6040 (1988), incorporated herein by reference for all purposes. Still other solid-phase techniques are described in U.S. Pat. No. 4,728,502 issued to Hamill and WO 90/00626 (Beattie, inventor).

Each of the above techniques produces only a relatively low density array of polymers. For example, the technique described in Geysen et al. is limited to producing 96 different polymers on pins spaced in the dimensions of a standard microtiter plate.

Improved methods of forming large arrays of oligonucleotides, peptides and other polymer sequences in a short period of time have been devised. Of particular note, Pirrung et al., U.S. Pat. No. 5,143,854 (see also PCT Application No. WO 90/15070) and Fodor et al., PCT Publication No. WO 92/10092, all incorporated herein by reference, disclose methods of forming vast arrays of peptides, oligonucleotides and other polymer sequences using, for example, light-directed synthesis techniques. See also, Fodor et al., *Science*, 251:767-777 (1991), also incorporated herein by reference for all purposes. These procedures are now referred to as VLSIPS™ procedures.

In the above-referenced Fodor et al., PCT application, an elegant method is described for using a computer-controlled system to direct a VLSIPS™ procedure. Using this approach, one heterogeneous array of polymers is converted, through simultaneous coupling at a number of reaction sites, into a different heterogeneous array. See, U.S. Pat. No. 5,384,261 and U.S. application Ser. No. 07/980,523, the disclosures of which are incorporated herein for all purposes.

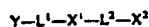
The development of VLSIPS™ technology as described in the above-noted U.S. Pat. No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092, is considered pioneering technology in the fields of combinatorial synthesis and screening of combinatorial libraries. More recently, patent application Ser. No. 08/082,937, filed Jun. 25, 1993 now abandoned, describes methods for making arrays of oligonucleotide probes that can be used to check or determine a partial or complete sequence of a target nucleic acid and to detect the presence of a nucleic acid containing a specific oligonucleotide sequence.

A number of biochemical processes of pharmaceutical interest involve the interaction of some species, e.g., a drug, a peptide or protein, or RNA, with double-stranded DNA. For example, protein/DNA binding interactions are involved with a number of transcription factors as well as tumor suppression associated with the p53 protein and the genes contributing to a number of cancer conditions.

SUMMARY OF THE INVENTION

High-density arrays of diverse unimolecular, double-stranded oligonucleotides, as well as arrays of conformationally restricted probes and methods for their use are provided by virtue of the present invention. In addition, methods and devices for detecting duplex formation of oligonucleotides on an array of diverse single-stranded oligonucleotides are also provided by this invention. Further, an adhesive based on the specific binding characteristics of two arrays of complementary oligonucleotides is provided in the present invention.

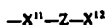
According to one aspect of the present invention, libraries of unimolecular, double-stranded oligonucleotides are provided. Each member of the library is comprised of a solid support, an optional spacer for attaching the double-stranded oligonucleotide to the support and for providing sufficient space between the double-stranded oligonucleotide and the solid support for subsequent binding studies and assays, an oligonucleotide attached to the spacer and further attached to a second complementary oligonucleotide by means of a flexible linker, such that the two oligonucleotide portions exist in a double-stranded configuration. More particularly, the members of the libraries of the present invention can be represented by the formula:



in which Y is a solid support, L¹ is a bond or a spacer, L² is a flexible linking group, and X¹ and X² are a pair of complementary oligonucleotides.

In a specific aspect of the invention, the library of different unimolecular, double-stranded oligonucleotides can be used for screening a sample for a species which binds to one or more members of the library.

In a related aspect of the invention, a library of different conformationally-restricted probes attached to a solid support is provided. The individual members each have the formula:



in which X^{11} and X^{12} are complementary oligonucleotides and Z is a probe having sufficient length such that X^{11} and X^{12} form a double-stranded oligonucleotide portion of the member and thereby restrict the conformations available to the probe. In a specific aspect of the invention, the library of different conformationally-restricted probes can be used for screening a sample for a species which binds to one or more probes in the library.

According to yet another aspect of the present invention, methods and devices for the bioelectronic detection of duplex formation are provided.

According to still another aspect of the invention, an adhesive is provided which comprises two surfaces of complementary oligonucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A to 1F illustrate the preparation of a member of a library of surface-bound, unimolecular double-stranded DNA as well as binding studies with receptors having specificity for either the double stranded DNA portion, a probe which is held in a conformationally restricted form by DNA scaffolding, or a bulge or loop region of RNA.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Abbreviations

The following abbreviations are used herein: phi, phenanthrenequinone diimine; phen', 5-amido-glutaric acid-1,10-phenanthroline; dppz, dipyrrophenazine.

Glossary

The following terms are intended to have the following general meanings as they are used herein:

Chemical terms: As used herein, the term "alkyl" refers to a saturated hydrocarbon radical which may be straight-chain or branched-chain (for example, ethyl, isopropyl, t-amyl, or 2,5-dimethylhexyl). When "alkyl" or "alkylene" is used to refer to a linking group or a spacer, it is taken to be a group having two available valences for covalent attachment, for example, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH(CH_3)CH_2-$ and $-CH_2(CH_2CH_2)_2CH_2-$. Preferred alkyl groups as substituents are those containing 1 to 10 carbon atoms, with those containing 1 to 6 carbon atoms being particularly preferred. Preferred alkyl or alkylene groups as linking groups are those containing 1 to 20 carbon atoms, with those containing 3 to 6 carbon atoms being particularly preferred. The term "polyethylene glycol" is used to refer to those molecules which have repeating units of ethylene glycol, for example, hexaethylene glycol ($HO-(CH_2CH_2O)_5-CH_2CH_2OH$). When the term "polyethylene glycol" is used to refer to linking groups and spacer groups, it would be understood by one of skill in the art that other polyethers or polyols could be used as well (i. e., polypropylene glycol or mixtures of ethylene and propylene glycols).

The term "protecting group" as used herein, refers to any of the groups which are designed to block one reactive site in a molecule while a chemical reaction is carried out at another reactive site. More particularly, the protecting groups used herein can be any of those groups described in Greene, et al., *Protective Groups In Organic Chemistry*, 2nd Ed., John Wiley & Sons, New York, N.Y., 1991, incorporated herein by reference. The proper selection of protecting groups for a particular synthesis will be governed by the overall methods employed in the synthesis. For example, in

"light-directed" synthesis, discussed below, the protecting groups will be photolabile protecting groups such as NVOC, MeNPOC, and those disclosed in co-pending Application PCT/US93/10162 (filed Oct. 22, 1993), incorporated herein by reference. In other methods, protecting groups may be removed by chemical methods and include groups such as FMOC, DMT and others known to those of skill in the art.

Complementary or substantially complementary: Refers to the hybridization or base pairing between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%.

Alternatively, substantial complementary exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanchisa *Nucleic Acids Res.* 12:203 (1984), incorporated herein by reference.

Stringent hybridization conditions will typically include salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM. Hybridization temperatures can be as low as 5° C., but are typically greater than 22° C., more typically greater than about 30° C., and preferably in excess of about 37° C. Longer fragments may require higher hybridization temperatures for specific hybridization. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone.

Epitope: The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.

Identifier tag: A means whereby one can identify which molecules have experienced a particular reaction in the synthesis of an oligomer. The identifier tag also records the step in the synthesis series in which the molecules experienced that particular monomer reaction. The identifier tag may be any recognizable feature which is, for example: microscopically distinguishable in shape, size, color, optical density, etc.; differently absorbing or emitting of light; chemically reactive; magnetically or electronically encoded; or in some other way distinctively marked with the required information. A preferred example of such an identifier tag is an oligonucleotide sequence.

Ligand/Probe: A ligand is a molecule that is recognized by a particular receptor. The agent bound by or reacting with a receptor is called a "ligand," a term which is definitionally meaningful only in terms of its counterpart receptor. The term "ligand" does not imply any particular molecular size or other structural or compositional feature other than that the substance in question is capable of binding or otherwise interacting with the receptor. Also, a ligand may serve either as the natural ligand to which the receptor binds, or as a functional analogue that may act as an agonist or antagonist.

Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, substrate analogs, transition state analogs, cofactors, drugs, proteins, and antibodies. The term "probe" refers to those molecules which are expected to act like ligands but for which binding information is typically unknown. For example, if a receptor is known to bind a ligand which is a peptide β -turn, a "probe" or library of probes will be those molecules designed to mimic the peptide β -turn. In instances where the particular ligand associated with a given receptor is unknown, the term probe refers to those molecules designed as potential ligands for the receptor.

Monomer: Any member of the set of molecules which can be joined together to form an oligomer or polymer. The set of monomers useful in the present invention includes, but is not restricted to, for the example of oligonucleotide synthesis, the set of nucleotides consisting of adenine, thymine, cytosine, guanine, and uridine (A, T, C, G, and U, respectively) and synthetic analogs thereof. As used herein, monomers refers to any member of a basis set for synthesis of an oligomer. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer.

Oligomer or Polymer: The oligomer or polymer sequences of the present invention are formed from the chemical or enzymatic addition of monomer subunits. Such oligomers include, for example, both linear, cyclic, and branched polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α -, β -, or ω -amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacrylates, or other polymers which will be readily apparent to one skilled in the art upon review of this disclosure. As used herein, the term oligomer or polymer is meant to include such molecules as β -turn mimetics, prostaglandins and benzodiazepines which can also be synthesized in a stepwise fashion on a solid support.

Peptide: A peptide is an oligomer in which the monomers are amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that when α -amino acids are used, they may be the L-optical isomer or the D-optical isomer. Other amino acids which are useful in the present invention include unnatural amino acids such as β -alanine, phenylglycine, homoarginine and the like. Peptides are more than two amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, *Biochemistry*, Third Ed., (1988), which is incorporated herein by reference for all purposes.

Oligonucleotides: An oligonucleotide is a single-stranded DNA or RNA molecule, typically prepared by synthetic means. Alternatively, naturally occurring oligonucleotides, or fragments thereof, may be isolated from their natural sources or purchased from commercial sources. Those oligonucleotides employed in the present invention will be 4 to 100 nucleotides in length, preferably from 6 to 30 nucleotides, although oligonucleotides of different length may be appropriate. Suitable oligonucleotides may be prepared by the phosphoramidite method described by Beaucage and Carruthers, *Tetrahedron Lett.*, 22:1859-1862 (1981), or by the triester method according to Matteucci, et al., *J. Am.*

Chem. Soc., 103:3185 (1981), both incorporated herein by reference, or by other chemical methods using either a commercial automated oligonucleotide synthesizer or VLSIPS™ technology (discussed in detail below). When oligonucleotides are referred to as "double-stranded," it is understood by those of skill in the art that a pair of oligonucleotides exist in a hydrogen-bonded, helical array typically associated with, for example, DNA. In addition to the 100% complementary form of double-stranded oligonucleotides, the term "double-stranded" as used herein is also meant to refer to those forms which include such structural features as bulges and loops, described more fully in such biochemistry texts as Stryer, *Biochemistry*, Third Ed., (1988), previously incorporated herein by reference for all purposes.

Receptor: A molecule that has an affinity for a given ligand or probe. Receptors may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered natural or isolated state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "ligand-receptor pair" is formed when two molecules have combined through molecular recognition to form a complex. Other examples of receptors which can be investigated by this invention include but are not restricted to:

- a) **Microorganism receptors:** Determination of ligands or probes that bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.
- b) **Enzymes:** For instance, the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters. Determination of ligands or probes that bind to certain receptors, and thus modulate the action of the enzymes that cleave the different neurotransmitters, is useful in the development of drugs that can be used in the treatment of disorders of neurotransmission.
- c) **Antibodies:** For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest. Determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences, or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for autoimmune diseases (e.g., by blocking the binding of the "self" antibodies).
- d) **Nucleic Acids:** The invention may be useful in investigating sequences of nucleic acids acting as binding sites for cellular proteins ("trans-acting factors"). Such sequences may include, e.g., transcription factors, suppressors, enhancers or promoter sequences.
- e) **Catalytic Polypeptides:** Polymers, preferably polypeptides, which are capable of promoting a chemical

reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, Lerner, R.A. et al., *Science* 252: 659 (1991), which is incorporated herein by reference.

f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone that can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands that bind to a receptor may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

Substrate or Solid Support: A material having a rigid or semi-rigid surface. Such materials will preferably take the form of plates or slides, small beads, pellets, disks or other convenient forms, although other forms may be used. In some embodiments, at least one surface of the substrate will be substantially flat. In other embodiments, a roughly spherical shape is preferred.

Synthetic: Produced by in vitro chemical or enzymatic synthesis. The synthetic libraries of the present invention may be contrasted with those in viral or plasmid vectors, for instance, which may be propagated in bacterial, yeast, or other living hosts.

DESCRIPTION OF THE INVENTION

The broad concept of the present invention is illustrated in FIGS. 1A to 1F. FIGS. 1A, 1B and 1C illustrate the preparation of surface-bound unimolecular double stranded DNA, while FIGS. 1D, 1E, and 1F illustrate uses for the libraries of the present invention.

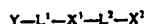
FIG. 1A shows a solid support 1 having an attached spacer 2, which is optional. Attached to the distal end of the spacer is a first oligomer 3, which can be attached as a single unit or synthesized on the support or spacer in a monomer by monomer approach. FIG. 1B shows a subsequent stage in the preparation of one member of a library according to the present invention. In this stage, a flexible linker 4 is attached to the distal end of the oligomer 3. In other embodiments, the flexible linker will be a probe. FIG. 1C shows the completed surface-bound unimolecular double stranded DNA which is one member of a library, wherein a second oligomer 5 is now attached to the distal end of the flexible linker (or probe). As shown in FIG. 1C, the length of the flexible linker (or probe) 4 is sufficient such that the first and second oligomers (which are complementary) exist in a double-stranded conformation. It will be appreciated by one of skill in the art, that the libraries of the present invention will contain multiple, individually synthesized members which can be screened for various types of activity. Three such binding events are illustrated in FIGS. 1D, 1E and 1F.

In FIG. 1D, a receptor 6, which can be a protein, RNA molecule or other molecule which is known to bind to DNA, is introduced to the library. Determining which member of a library binds to the receptor provides information which is useful for diagnosing diseases, sequencing DNA or RNA, identifying genetic characteristics, or in drug discovery.

In FIG. 1E, the linker 4 is a probe for which binding information is sought. The probe is held in a conformationally restricted manner by the flanking oligomers 3 and 5, which are present in a double-stranded conformation. As a result, a library of conformationally restricted probes can be screened for binding activity with a receptor 7 which has specificity for the probe.

The present invention also contemplates the preparation of libraries of unimolecular, double-stranded oligonucleotides having bulges or loops in one of the strands as depicted in FIG. 1F. In FIG. 1F, one oligonucleotide 5 is shown as having a bulge 8. Specific RNA bulges are often recognized by proteins (e.g., TAR RNA is recognized by the TAT protein of HIV). Accordingly, libraries of RNA bulges or loops are useful in a number of diagnostic applications. One of skill in the art will appreciate that the bulge or loop can be present in either oligonucleotide portion 3 or 5. Libraries of Unimolecular, Double-Stranded Oligonucleotides

In one aspect, the present invention provides libraries of unimolecular double-stranded oligonucleotides, each member of the library having the formula:



in which Y represents a solid support, X^1 and X^2 represent a pair of complementary oligonucleotides, L^1 represents a bond or a spacer, and L^2 represents a linking group having sufficient length such that X^1 and X^2 form a double-stranded oligonucleotide.

The solid support may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The solid support is preferably flat but may take on alternative surface configurations. For example, the solid support may contain raised or depressed regions on which synthesis takes place. In some embodiments, the solid support will be chosen to provide appropriate light-absorbing characteristics. For example, the support may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2 , SiN_x , modified silicon, or any one of a variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidene difluoride, polystyrene, polycarbonate, or combinations thereof. Other suitable solid support materials will be readily apparent to those of skill in the art. Preferably, the surface of the solid support will contain reactive groups, which could be carboxyl, amino, hydroxyl, thiol, or the like. More preferably, the surface will be optically transparent and will have surface Si—OH functionalities, such as are found on silica surfaces.

Attached to the solid support is an optional spacer, L^1 . The spacer molecules are preferably of sufficient length to permit the double-stranded oligonucleotides in the completed member of the library to interact freely with molecules exposed to the library. The spacer molecules, when present, are typically 6–50 atoms long to provide sufficient exposure for the attached double-stranded DNA molecule. The spacer, L^1 , is comprised of a surface attaching portion and a longer chain portion. The surface attaching portion is that part of L^1 which is directly attached to the solid support. This portion

can be attached to the solid support via carbon-carbon bonds using, for example, supports having (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide as the solid support). Siloxane bonds with the surface of the support are formed in one embodiment via reactions of surface attaching portions bearing trichlorosilyl or trialkoxysilyl groups. The surface attaching groups will also have a site for attachment of the longer chain portion. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl. Preferred surface attaching portions include aminoalkylsilanes and hydroxyalkylsilanes. In particularly preferred embodiments, the surface attaching portion of L^1 is either bis(2-hydroxyethyl)-aminopropyltriethoxysilane, 2-hydroxyethylaminopropyltriethoxysilane, aminopropyltriethoxysilane or hydroxypropyltriethoxysilane.

The longer chain portion can be any of a variety of molecules which are inert to the subsequent conditions for polymer synthesis. These longer chain portions will typically be aryl acetylene, ethylene glycol oligomers containing 2-14 monomer units, diamines, diacids, amino acids, peptides, or combinations thereof. In some embodiments, the longer chain portion is a polynucleotide. The longer chain portion which is to be used as part of L^1 can be selected based upon its hydrophilic/hydrophobic properties to improve presentation of the double-stranded oligonucleotides to certain receptors, proteins or drugs. The longer chain portion of L^1 can be constructed of polyethyleneglycols, polynucleotides, alkylene, polyalcohol, polyester, polyamine, polyphosphodiester and combinations thereof. Additionally, for use in synthesis of the libraries of the invention, L^1 will typically have a protecting group, attached to a functional group (i.e., hydroxyl, amino or carboxylic acid) on the distal or terminal end of the chain portion (opposite the solid support). After deprotection and coupling, the distal end is covalently bound to an oligomer.

Attached to the distal end of L^1 is an oligonucleotide, X^1 , which is a single-stranded DNA or RNA molecule. The oligonucleotides which are part of the present invention are typically of from about 4 to about 100 nucleotides in length. Preferably, X^1 is an oligonucleotide which is about 6 to about 30 nucleotides in length. The oligonucleotide is typically linked to L^1 via the 3'-hydroxyl group of the oligonucleotide and a functional group on L^1 which results in the formation of an ether, ester, carbamate or phosphate ester linkage.

Attached to the distal end of X^1 is a linking group, L^2 , which is flexible and of sufficient length that X^1 can effectively hybridize with X^2 . The length of the linker will typically be a length which is at least the length spanned by two nucleotide monomers, and preferably at least four nucleotide monomers, while not be so long as to interfere with either the pairing of X^1 and X^2 or any subsequent assays. The linking group itself will typically be an alkylene group (of from about 6 to about 24 carbons in length), a polyethyleneglycol group (of from about 2 to about 24 ethyleneglycol monomers in a linear configuration), a polyalcohol group, a polyamine group (e.g., spermine, spermidine and polymeric derivatives thereof), a polyester group (e.g., poly(ethyl acrylate) having of from 3 to 15 ethyl acrylate monomers in a linear configuration), a polyphosphodiester group, or a polynucleotide (having from about 2 to about 12 nucleic acids). Preferably, the linking group will be a polyethyleneglycol group which is at least a tetraethyleneglycol, and more preferably, from about 1 to 4 hexaethyleneglycols linked in a linear array. For use in synthesis

of the compounds of the invention, the linking group will be provided with functional groups which can be suitably protected or activated. The linking group will be covalently attached to each of the complementary oligonucleotides, X^1 and X^2 , by means of an ether, ester, carbamate, phosphate ester or amine linkage. The flexible linking group L^2 will be attached to the 5'-hydroxyl of the terminal monomer of X^1 and to the 3'-hydroxyl of the initial monomer of X^2 . Preferred linkages are phosphate ester linkages which can be formed in the same manner as the oligonucleotide linkages which are present in X^1 and X^2 . For example, hexaethyleneglycol can be protected on one terminus with a photolabile protecting group (i.e., NVOC or MeNPOC) and activated on the other terminus with 2-cyanoethyl-N,N-diisopropylamino-chlorophosphite to form a phosphoramidite. This linking group can then be used for construction of the libraries in the same manner as the photolabile-protected, phosphoramidite-activated nucleotides. Alternatively, ester linkages to X^1 and X^2 can be formed when the L^2 has terminal carboxylic acid moieties (using the 5'-hydroxyl of X^1 and the 3'-hydroxyl of X^2). Other methods of forming ether, carbamate or amine linkages are known to those of skill in the art and particular reagents and references can be found in such texts as March, *Advanced Organic Chemistry*, 4th Ed., Wiley-Interscience, New York, N.Y., 1992, incorporated herein by reference.

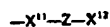
The oligonucleotide, X^2 , which is covalently attached to the distal end of the linking group is, like X^1 , a single-stranded DNA or RNA molecule. The oligonucleotides which are part of the present invention are typically of from about 4 to about 100 nucleotides in length. Preferably, X^2 is an oligonucleotide which is about 6 to about 30 nucleotides in length and exhibits complementary to X^1 of from 90 to 100%. More preferably, X^1 and X^2 are 100% complementary. In one group of embodiments, either X^1 or X^2 will further comprise a bulge or loop portion and exhibit complementary of from 90 to 100% over the remainder of the oligonucleotide.

In a particularly preferred embodiment, the solid support is a silica support, the spacer is a polyethyleneglycol conjugated to an aminoalkylsilane, the linking group is a polyethyleneglycol group, and X^1 and X^2 are complementary oligonucleotides each comprising of from 6 to 30 nucleic acid monomers.

The library can have virtually any number of different members, and will be limited only by the number or variety of compounds desired to be screened in a given application and by the synthetic capabilities of the practitioner. In one group of embodiments, the library will have from 2 up to 100 members. In other groups of embodiments, the library will have between 100 and 10000 members, and between 10000 and 1000000 members, preferably on a solid support. In preferred embodiments, the library will have a density of more than 100 members at known locations per cm^2 , preferably more than 1000 per cm^2 , more preferably more than 10,000 per cm^2 .

Libraries of Conformationally Restricted Probes

In still another aspect, the present invention provides libraries of conformationally-restricted probes. Each of the members of the library comprises a solid support having an optional spacer which is attached to an oligomer of the formula:



in which X^{11} and X^{12} are complementary oligonucleotides and Z is a probe. The probe will have sufficient length such that X^{11} and X^{12} form a double-stranded DNA portion of

each member. X^{11} and X^{12} are as described above for X^1 and X^2 respectively, except that for the present aspect of the invention, each member of the probe library can have the same X^{11} and the same X^{12} , and differ only in the probe portion. In one group of embodiments, X^{11} and X^{12} are either a poly-A oligonucleotide or a poly-T oligonucleotide.

As noted above, each member of the library will typically have a different probe portion. The probes, Z, can be any of a variety of structures for which receptor-probe binding information is sought for conformationally-restricted forms. For example, the probe can be an agonist or antagonist for a cell membrane receptor, a toxin, venom, viral epitope, hormone, peptide, enzyme, collector, drug, protein or antibody. In one group of embodiments, the probes are different peptides, each having of from about 4 to about 12 amino acids. Preferably the probes will be linked via polyphosphate diesters, although other linkages are also suitable. For example, the last monomer employed on the X^{11} chain can be a 5'-aminopropyl-functionalized phosphoramidite nucleotide (available from Glen Research, Sterling, Va., USA or Genosys Biotechnologies, The Woodlands, Tex., USA) which will provide a synthesis initiation site for the carboxy to amino synthesis of the peptide probe. Once the peptide probe is formed, a 3'-succinylated nucleoside (from Cruachem, Sterling, Va., USA) will be added under peptide coupling conditions. In yet another group of embodiments, the probes will be oligonucleotides of from 4 to about 30 nucleic acid monomers which will form a DNA or RNA hairpin structure. For use in synthesis, the probes can also have associated functional groups (i.e., hydroxyl, amino, carboxylic acid, anhydride and derivatives thereof) for attaching two positions on the probe to each of the complementary oligonucleotides.

The surface of the solid support is preferably provided with a spacer molecule, although it will be understood that the spacer molecules are not elements of this aspect of the invention. Where present, the spacer molecules will be as described above for L^1 .

The libraries of conformationally restricted probes can also have virtually any number of members. As above, the number of members will be limited only by design of the particular screening assay for which the library will be used, and by the synthetic capabilities of the practitioner. In one group of embodiments, the library will have from 2 to 100 members. In other groups of embodiments, the library will have between 100 and 10000 members, and between 10000 and 1000000 members. Also as above, in preferred embodiments, the library will have a density of more than 100 members at known locations per cm^2 , preferably more than 1000 per cm^2 , more preferably more than 10,000 per cm^2 .

Preparation of the Libraries

The present invention further provides methods for the preparation of diverse unimolecular, double-stranded oligonucleotides on a solid support. In one group of embodiments, the surface of a solid support has a plurality of preselected regions. An oligonucleotide of from 6 to 30 monomers is formed on each of the preselected regions. A linking group is then attached to the distal end of each of the oligonucleotides. Finally, a second oligonucleotide is formed on the distal end of each linking group such that the second oligonucleotide is complementary to the oligonucleotide already present in the same preselected region. The linking group used will have sufficient length such that the complementary oligonucleotides form a unimolecular, double-stranded oligonucleotide. In another group of embodiments, each chemically distinct member of the library will be synthesized on a separate solid support.

Libraries on a Single Substrate

Light-Directed Methods

For those embodiments using a single solid support, the oligonucleotides of the present invention can be formed using a variety of techniques known to those skilled in the art of polymer synthesis on solid supports. For example, "light directed" methods (which are one technique in a family of methods known as VLSIPS™ methods) are described in U.S. Pat. No. 5,143,854, previously incorporated by reference. The light directed methods discussed in the '854 patent involve activating predefined regions of a substrate or solid support and then contacting the substrate with a preselected monomer solution. The predefined regions can be activated with a light source, typically shown through a mask (much in the manner of photolithography techniques used in integrated circuit fabrication). Other regions of the substrate remain inactive because they are blocked by the mask from illumination and remain chemically protected. Thus, a light pattern defines which regions of the substrate react with a given monomer. By repeatedly activating different sets of predefined regions and contacting different monomer solutions with the substrate, a diverse array of polymers is produced on the substrate. Of course, other steps such as washing unreacted monomer solution from the substrate can be used as necessary. Other techniques include mechanical techniques such as those described in PCT No. 92/10183, U.S. Pat. No. 5,384,261 also incorporated herein by reference for all purposes. Still further techniques include bead based techniques such as those described in PCT US/93/04145, also incorporated herein by reference, and pin based methods such as those described in U.S. Pat. No. 5,288,514, also incorporated herein by reference.

The VLSIPS™ methods are preferred for making the compounds and libraries of the present invention. The surface of a solid support, optionally modified with spacers having photolabile protecting groups such as NVOC and MeNPOC, is illuminated through a photolithographic mask, yielding reactive groups (typically hydroxyl groups) in the illuminated regions. A 3'-O-phosphoramidite activated deoxynucleoside (protected at the 5'-hydroxyl with a photolabile protecting group) is then presented to the surface and chemical coupling occurs at sites that were exposed to light. Following capping, and oxidation, the substrate is rinsed and the surface illuminated through a second mask, to expose additional hydroxyl groups for coupling. A second 5'-protected, 3'-O-phosphoramidite activated deoxynucleoside is presented to the surface. The selective photodeprotection and coupling cycles are repeated until the desired set of oligonucleotides is produced. Alternatively, an oligomer of from, for example, 4 to 30 nucleotides can be added to each of the preselected regions rather than synthesize each member in a monomer by monomer approach. At this point in the synthesis, either a flexible linking group or a probe can be attached in a similar manner. For example, a flexible linking group such as polyethylene glycol will typically have an activating group (i.e., a phosphoramidite) on one end and a photolabile protecting group attached to the other end. Suitably derivatized polyethylene glycol linking groups can be prepared by the methods described in Durand, et al. *Nucleic Acids Res.* 18:6353-6359 (1990). Briefly, a polyethylene glycol (i.e., hexaethylene glycol) can be mono-protected using MeNPOC-chloride. Following purification of the mono-protected glycol, the remaining hydroxy moiety can be activated with 2-cyanoethyl-N,N-diisopropylaminochlorophosphate. Once the flexible linking group has been attached to the first oligonucleotide (X^1), deprotection and

coupling cycles will proceed using 5'-protected, 3'-O-phosphoramidite activated deoxynucleosides or intact oligomers. Probes can be attached in a manner similar to that used for the flexible linking group. When the desired probe is itself an oligomer, it can be formed either in stepwise fashion on the immobilized oligonucleotide or it can be separately synthesized and coupled to the immobilized oligomer in a single step. For example, preparation of conformationally restricted β -turn mimetics will typically involve synthesis of an oligonucleotide as described above, in which the last nucleoside monomer will be derivatized with an aminoalkyl-functionalized phosphoramidite. See, U.S. Pat. No. 5,288,514, previously incorporated by reference. The desired peptide probe is typically formed in the direction from carboxyl to amine terminus. Subsequent coupling of a 3'-succinylated nucleoside, for example, provides the first monomer in the construction of the complementary oligonucleotide strand (which is carried out by the above methods). Alternatively, a library of probes can be prepared by first derivatizing a solid support with multiple poly(A) or poly(T) oligonucleotides which are suitably protected with photolabile protecting groups, deprotecting at known sites and constructing the probe at those sites, then coupling the complementary poly(T) or poly(A) oligonucleotide.

Flow Channel or Spotting Methods

Additional methods applicable to library synthesis on a single substrate are described in co-pending applications Ser. No. 07/980,523, filed Nov. 20, 1992, and U.S. Pat. No. 5,384,261, incorporated herein by reference for all purposes. In the methods disclosed in these applications, reagents are delivered to the substrate by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. However, other approaches, as well as combinations of spotting and flowing, may be employed. In each instance, certain activated regions of the substrate are mechanically separated from other regions when the monomer solutions are delivered to the various reaction sites.

A typical "flow channel" method applied to the compounds and libraries of the present invention can generally be described as follows. Diverse polymer sequences are synthesized at selected regions of a substrate or solid support by forming flow channels on a surface of the substrate through which appropriate reagents flow or in which appropriate reagents are placed. For example, assume a monomer "A" is to be bound to the substrate in a first group of selected regions. If necessary, all or part of the surface of the substrate in all or a part of the selected regions is activated for binding by, for example, flowing appropriate reagents through all or some of the channels, or by washing the entire substrate with appropriate reagents. After placement of a channel block on the surface of the substrate, a reagent having the monomer A flows through or is placed in all or some of the channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the substrate directly or indirectly (via a spacer) in the first selected regions.

Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or replacement of the channel block on the surface of the substrate; through opening or closing a selected valve; or through deposition of a layer of chemical or photoresist. If necessary, a step is performed for activating at least the second regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer B at the second selected locations. In this particu-

lar example, the resulting sequences bound to the substrate at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of sequences of desired length at known locations on the substrate.

After the substrate is activated, monomer A can be flowed through some of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the substrate must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can be minimized.

One of skill in the art will recognize that there are alternative methods of forming channels or otherwise protecting a portion of the surface of the substrate. For example, according to some embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

The "spotting" methods of preparing compounds and libraries of the present invention can be implemented in much the same manner as the flow channel methods. For example, a monomer A can be delivered to and coupled with a first group of reaction regions which have been appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively small quantities of them in selected regions. In some steps, of course, the entire substrate surface can be sprayed or otherwise coated with a solution. In preferred embodiments, a dispenser moves from region to region, depositing only as much monomer as necessary at each stop. Typical dispensers include a micropipette to deliver the monomer solution to the substrate and a robotic system to control the position of the micropipette with respect to the substrate, or an ink-jet printer. In other embodiments, the dispenser includes a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions simultaneously.

Pin-Based Methods

Another method which is useful for the preparation of compounds and libraries of the present invention involves "pin based synthesis." This method is described in detail in U.S. Pat. No. 5,288,514, previously incorporated herein by reference. The method utilizes a substrate having a plurality of pins or other extensions. The pins are each inserted simultaneously into individual reagent containers in a tray. In a common embodiment, an array of 96 pins/containers is utilized.

Each tray is filled with a particular reagent for coupling in a particular chemical reaction on an individual pin. Accordingly, the trays will often contain different reagents. Since the chemistry disclosed herein has been established such that a relatively similar set of reaction conditions may be utilized to perform each of the reactions, it becomes possible to conduct multiple chemical coupling steps simultaneously. In the first step of the process the invention provides for the use of substrate(s) on which the chemical coupling steps are conducted. The substrate is optionally provided with a

spacer having active sites. In the particular case of oligonucleotides, for example, the spacer may be selected from a wide variety of molecules which can be used in organic environments associated with synthesis as well as aqueous environments associated with binding studies. Examples of suitable spacers are polyethyleneglycols, dicarboxylic acids, polyamines and alkenes, substituted with, for example, methoxy and ethoxy groups. Additionally, the spacers will have an active site on the distal end. The active sites are optionally protected initially by protecting groups. Among a wide variety of protecting groups which are useful are Fmoc, Boc, t-butyl esters, t-butyl ethers, and the like. Various exemplary protecting groups are described in, for example, Atherton et al., *Solid Phase Peptide Synthesis*, IRL Press (1989), incorporated herein by reference. In some embodiments, the spacer may provide for a cleavable function by way of, for example, exposure to acid or base.

Libraries on Multiple Substrates

Bead Based Methods

Yet another method which is useful for synthesis of compounds and libraries of the present invention involves "bead based synthesis." A general approach for bead based synthesis is described in copending application Ser. Nos. 07/762,522 (filed Sep. 18, 1991 now abandoned); 07/946,239 (filed Sep. 16, 1992); 08/146,886 (filed Nov. 2, 1993); 07/876,792 (filed Apr. 29, 1992) and PCT/US93/04145 (filed Apr. 28, 1993), the disclosures of which are incorporated herein by reference.

For the synthesis of molecules such as oligonucleotides on beads, a large plurality of beads are suspended in a suitable carrier (such as water) in a container. The beads are provided with optional spacer molecules having an active site. The active site is protected by an optional protecting group.

In a first step of the synthesis, the beads are divided for coupling into a plurality of containers. For the purposes of this brief description, the number of containers will be limited to three, and the monomers denoted as A, B, C, D, E, and F. The protecting groups are then removed and a first portion of the molecule to be synthesized is added to each of the three containers (i. e., A is added to container 1, B is added to container 2 and C is added to container 3).

Thereafter, the various beads are appropriately washed of excess reagents, and remixed in one container. Again, it will be recognized that by virtue of the large number of beads utilized at the outset, there will similarly be a large number of beads randomly dispersed in the container, each having a particular first portion of the monomer to be synthesized on a surface thereof.

Thereafter, the various beads are again divided for coupling in another group of three containers. The beads in the first container are deprotected and exposed to a second monomer (D), while the beads in the second and third containers are coupled to molecule portions E and F respectively. Accordingly, molecules AD, BD, and CD will be present in the first container, while AE, BE, and CE will be present in the second container, and molecules AF, BF, and CF will be present in the third container. Each bead, however, will have only a single type of molecule on its surface. Thus, all of the possible molecules formed from the first portions A, B, C, and the second portions D, E, and F have been formed.

The beads are then recombined into one container and additional steps such as are conducted to complete the synthesis of the polymer molecules. In a preferred embodiment, the beads are tagged with an identifying tag which is unique to the particular double-stranded oligonucleotide or

probe which is present on each bead. A complete description of identifier tags for use in synthetic libraries is provided in co-pending application Ser. No. 08/146,886 (filed Nov. 2, 1993) previously incorporated by reference for all purposes.

Methods of Library Screening

A library prepared according to any of the methods described above can be used to screen for receptors having high affinity for either unimolecular, double-stranded oligonucleotides or conformationally restricted probes. In one group of embodiments, a solution containing a marked (labelled) receptor is introduced to the library and incubated for a suitable period of time. The library is then washed free of unbound receptor and the probes or double-stranded oligonucleotides having high affinity for the receptor are identified by identifying those regions on the surface of the library where markers are located. Suitable markers include, but are not limited to, radiolabels, chromophores, fluorophores, chemiluminescent moieties, and transition metals. Alternatively, the presence of receptors may be detected using a variety of other techniques, such as an assay with a labelled enzyme, antibody, and the like. Other techniques using various marker systems for detecting bound receptor will be readily apparent to those skilled in the art.

In a preferred embodiment, a library prepared on a single solid support (using, for example, the VLSIPS™ technique) can be exposed to a solution containing marked receptor such as a marked antibody. The receptor can be marked in any of a variety of ways, but in one embodiment marking is effected with a radioactive label. The marked antibody binds with high affinity to an immobilized antigen previously localized on the surface. After washing the surface free of unbound receptor, the surface is placed proximate to x-ray film or phosphorimagers to identify the antigens that are recognized by the antibody. Alternatively, a fluorescent marker may be provided and detection may be by way of a charge-coupled device (CCD), fluorescence microscopy or laser scanning.

When autoradiography is the detection method used, the marker is a radioactive label, such as ³²P. The marker on the surface is exposed to X-ray film or a phosphorimager, which is developed and read out on a scanner. An exposure time of about 1 hour is typical in one embodiment. Fluorescence detection using a fluorophore label, such as fluorescein, attached to the receptor will usually require shorter exposure times.

Quantitative assays for receptor concentrations can also be performed according to the present invention. In a direct assay method, the surface containing localized probes prepared as described above, is incubated with a solution containing a marked receptor for a suitable period of time. The surface is then washed free of unbound receptor. The amount of marker present at predefined regions of the surface is then measured and can be related to the amount of receptor in solution. Methods and conditions for performing such assays are well-known and are presented in, for example, L. Hood et al., *Immunology*, Benjamin/Cummings (1978), and E. Harlow et al., *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988). See, also U.S. Pat. No. 4,376,110 for methods of performing sandwich assays. The precise conditions for performing these steps will be apparent to one skilled in the art.

A competitive assay method for two receptors can also be employed using the present invention. Methods of conducting competitive assays are known to those of skill in the art. One such method involves immobilizing conformationally restricted probes on predefined regions of a surface as described above. An unmarked first receptor is then bound

to the probes on the surface having a known specific binding affinity for the receptors. A solution containing a marked second receptor is then introduced to the surface and incubated for a suitable time. The surface is then washed free of unbound reagents and the amount of marker remaining on the surface is measured. In another form of competition assay, marked and unmarked receptors can be exposed to the surface simultaneously. The amount of marker remaining on predefined regions of the surface can be related to the amount of unknown receptor in solution. Yet another form of competition assay will utilize two receptors having different labels, for example, two different chromophores.

In other embodiments, in order to detect receptor binding, the double-stranded oligonucleotides which are formed with attached probes or with a flexible linking group will be treated with an intercalating dye, preferably a fluorescent dye. The library can be scanned to establish a background fluorescence. After exposure of the library to a receptor solution, the exposed library will be scanned or illuminated and examined for those areas in which fluorescence has changed. Alternatively, the receptor of interest can be labeled with a fluorescent dye by methods known to those of skill in the art and incubated with the library of probes. The library can then be scanned or illuminated, as above, and examined for areas of fluorescence.

In instances where the libraries are synthesized on beads in a number of containers, the beads are exposed to a receptor of interest. In a preferred embodiment the receptor is fluorescently or radioactively labelled. Thereafter, one or more beads are identified that exhibit significant levels of, for example, fluorescence using one of a variety of techniques. For example, in one embodiment, mechanical separation under a microscope is utilized. The identity of the molecule on the surface of such separated beads is then identified using, for example, NMR, mass spectrometry, PCR amplification and sequencing of the associated DNA, or the like. In another embodiment, automated sorting (i.e., fluorescence activated cell sorting) can be used to separate beads (bearing probes) which bind to receptors from those which do not bind. Typically the beads will be labeled and identified by methods disclosed in Needels, et al., *Proc. Natl. Acad. Sci., USA* 90:10700-10704 (1993), incorporated herein by reference.

The assay methods described above for the libraries of the present invention will have tremendous application in such endeavors as DNA "footprinting" of proteins which bind DNA. Currently, DNA footprinting is conducted using DNase I digestion of double-stranded DNA in the presence of a putative DNA binding protein. Gel analysis of cut and protected DNA fragments then provides a "footprint" of where the protein contacts the DNA. This method is both labor and time intensive. See, Galas et al., *Nucleic Acid Res.* 5:3157 (1978). Using the above methods, a "footprint" could be produced using a single array of unimolecular, double-stranded oligonucleotides in a fraction of the time of conventional methods. Typically, the protein will be labeled with a radioactive or fluorescent species and incubated with a library of unimolecular, double-stranded DNA. Phosphorimaging or fluorescence detection will provide a footprint of those regions on the library where the protein has bound. Alternatively, unlabeled protein can be used. When unlabeled protein is used, the double-stranded oligonucleotides in the library will all be labeled with a marker, typically a fluorescent marker. Incorporation of a marker into each member of the library can be carried out by terminating the oligonucleotide synthesis with a commercially available fluorescing phosphoramidite nucleotide derivative. Follow-

ing incubation with the unlabeled protein, the library will be treated with DNase I and examined for areas which are protected from cleavage.

The assay methods described above for the libraries of the present invention can also be used in reverse drug discovery. In such an application, a compound having known pharmacological safety or other desired properties (e.g., aspirin) could be screened against a variety of double-stranded oligonucleotides for potential binding. If the compound is shown to bind to a sequence associated with, for example, tumor suppression, the compound can be further examined for efficacy in the related diseases.

In other embodiments, probe arrays comprising β -turn mimetics can be prepared and assayed for activity against a particular receptor. β -turn mimetics are compounds having molecular structures similar to β -turns which are one of the three major components in protein molecular architecture. β -turns are similar in concept to hairpin turns of oligonucleotide strands, and are often critical recognition features for various protein-ligand and protein-protein interactions. As a result, a library of β -turn mimetic probes can provide or suggest new therapeutic agents having a particular affinity for a receptor which will correspond to the affinity exhibited by the β -turn and its receptor.

25 Bioelectronic Devices and Methods

In another aspect, the present invention provides a method for the bioelectronic detection of sequence-specific oligonucleotide hybridization. A general method and device which is useful in diagnostics in which a biochemical species is attached to the surface of a sensor is described in U.S. Pat. No. 4,562,157 (the Lowe patent), incorporated herein by reference. The present method utilizes arrays of immobilized oligonucleotides (prepared, for example, using VLSIPS™ technology) and the known photo-induced electron transfer which is mediated by a DNA double helix structure. See, Murphy et al., *Science* 262:1025-1029 (1993). This method is useful in hybridization-based diagnostics, as a replacement for fluorescence-based detection systems. The method of bioelectronic detection also offers higher resolution and potentially higher sensitivity than earlier diagnostic methods involving sequencing/detecting by hybridization. As a result, this method finds applications in genetic mutation screening and primary sequencing of oligonucleotides. The method can also be used for Sequencing By Hybridization (SBH), which is described in co-pending application Ser. Nos. 08/082,937 (filed Jun. 25, 1993 now abandoned) and 08/168,904 (filed Dec. 15, 1993), each of which are incorporated herein by reference for all purposes. This method uses a set of short oligonucleotide probes of defined sequence to search for complementary sequences on a longer target strand of DNA. The hybridization pattern is used to reconstruct the target DNA sequence. Thus, the hybridization analysis of large numbers of probes can be used to sequence long stretches of DNA. In immediate applications of this hybridization methodology, a small number of probes can be used to interrogate local DNA sequence.

In the present inventive method, hybridization is monitored using bioelectronic detection. In this method, the target DNA, or first oligonucleotide, is provided with an electron-donor tag and then incubated with an array of oligonucleotide probes, each of which bears an electron-acceptor tag and occupies a known position on the surface of the array. After hybridization of the first oligonucleotide to the array has occurred, the hybridized array is illuminated to induce an electron transfer reaction in the direction of the surface of the array. The electron transfer reaction is then detected at

the location on the surface where hybridization has taken place. Typically, each of the oligonucleotide probes in an array will have an attached electron-acceptor tag located near the surface of the solid support used in preparation of the array. In embodiments in which the arrays are prepared by light-directed methods (i.e., typically 3' to 5' direction), the electron-acceptor tag will be located near the 3' position. The electron-acceptor tag can be attached either to the 3' monomer by methods known to those of skill in the art, or it can be attached to a spacing group between the 3' monomer and the solid support. Such a spacing group will have, in addition to functional groups for attachment to the solid support and the oligonucleotide, a third functional group for attachment of the electron-acceptor tag. The target oligonucleotide will typically have the electron-donor tag attached at the 3' position. Alternatively, the target oligonucleotide can be incubated with the array in the absence of an electron-donor tag. Following incubation, the electron-donor tag can be added in solution. The electron-donor tag will then intercalate into those regions where hybridization has occurred. An electron transfer reaction can then be detected in those regions having a continuous DNA double helix.

The electron-donor tag can be any of a variety of complexes which participate in electron transfer reactions and which can be attached to an oligonucleotide by a means which does not interfere with the electron transfer reaction. In preferred embodiments, the electron-donor tag is a ruthenium (II) complex, more preferably a ruthenium (II) (phen)₂(dppz) complex.

The electron-acceptor tag can be any species which, with the electron-donor tag, will participate in an electron transfer reaction. An example of an electron-acceptor tag is a rhodium (III) complex. A preferred electron-acceptor tag is a rhodium (III) (phen)₂(phen') complex.

In a particularly preferred embodiment, the electron-donor tag is a ruthenium (II) (phen)₂(dppz) complex and the electron-acceptor tag is a rhodium (III) (phen)₂(phen') complex.

In still another aspect, the present invention provides a device for the bioelectronic detection of sequence-specific oligonucleotide hybridization. The device will typically consist of a sensor having a surface to which an array of oligonucleotides are attached. The oligonucleotides will be attached in pre-defined areas on the surface of the sensor and have an electron-acceptor tag attached to each oligonucleotide. The electron-acceptor tag will be a tag which is capable of producing an electron transfer signal upon illumination of a hybridized species, when the complementary oligonucleotide bears an electron-donating tag. The signal will be in the direction of the sensor surface and be detected by the sensor.

In a preferred embodiment, the sensor surface will be a silicon-based surface which can sense the electronic signal induced and, if necessary, amplify the signal. The metal contacts on which the probes will be synthesized can be treated with an oxygen plasma prior to synthesis of the probes to enhance the silane adhesion and concentration on the surface. The surface will further comprise a multi-gated field effect transistor, with each gate serving as a sensor and different oligonucleotides attached to each gate. The oligonucleotides will typically be attached to the metal contacts on the sensor surface by means of a spacer group.

The spacer group should not be too long, in order to ensure that the sensing function of the device is easily activated by the binding interaction and subsequent illumination of the "tagged" hybridized oligonucleotides. Prefer-

ably, the spacer group is from 3 to 12 atoms in length and will be as described above for the surface modifying portion of the spacer group, L¹.

The oligonucleotides which are attached to the spacer group can be formed by any of the solid phase techniques which are known to those of skill in the art. Preferably, the oligonucleotides are formed one base at a time in the direction of the 3' terminus to the 5' terminus by the "light-directed" methods described above. The oligonucleotide can then be modified at the 3' end to attach the electron-acceptor tag. A number of suitable methods of attachment are known. For example, modification with the reagent Aminolink2 (from Applied Biosystems, Inc.) provides a terminal phosphate moiety which is derivatized with an aminobenzyl phosphate ester. Coupling of a carboxylic acid, which is present on the electron-acceptor tag, to the amine can then be carried out using HOBt and DCC. Alternatively, synthesis of the oligonucleotide can begin with a suitably derivatized and protected monomer which can then be deprotected and coupled to the electron-acceptor tag once the complete oligonucleotide has been synthesized.

The silica surface can also be replaced by silicon nitride or oxynitride, or by an oxide of another metal, especially aluminum, titanium (IV) or iron (III). The surface can also be any other film, membrane, insulator or semiconductor overlying the sensor which will not interfere with the detection of electron transfer and to which an oligonucleotide can be coupled.

Additionally, detection devices other than an FET can be used. For example, sensors such as bipolar transistors, MOS transistors and the like are also useful for the detection of electron transfer signals.

Adhesives

In still another aspect, the present invention provides an adhesive comprising a pair of surfaces, each having a plurality of attached oligonucleotides, wherein the single-stranded oligonucleotides on one surface are complementary to the single-stranded oligonucleotides on the other surface. The strength and position/orientation specificity can be controlled using a number of factors including the number and length of oligonucleotides on each surface, the degree of complementarity, and the spatial arrangement of complementary oligonucleotides on the surface. For example, increasing the number and length of the oligonucleotides on each surface will provide a stronger adhesive. Suitable lengths of oligonucleotides are typically from about 10 to about 70 nucleotides. Additionally, the surfaces of oligonucleotides can be prepared such that adhesion occurs in an extremely position-specific manner by a suitable arrangement of complementary oligonucleotides in a specific pattern. Small deviations from the optimum spatial arrangement are energetically unfavorable as many hybridization bonds must be broken and are not reformed in any other relative orientation.

The adhesives of the present invention will find use in numerous applications. Generally, the adhesives are useful for adhering two surfaces to one another. More specifically, the adhesives will find application where biological compatibility of the adhesive is desired. An example of a biological application involves use in surgical procedures where tissues must be held in fixed positions during or following the procedure. In this application, the surfaces of the adhesive will typically be membranes which are compatible with the tissues to which they are attached.

A particular advantage of the adhesives of the present invention is that when they are formed in an orientation specific manner, the adhesive portions will be "self-finding."

that is the system will go to the thermodynamic equilibrium in which the two sides are matched in the predetermined, orientation specific manner.

EXAMPLES

Example 1

This example illustrates the general synthesis of an array of unimolecular, double-stranded oligonucleotides on a solid support.

Unimolecular double stranded DNA molecules were synthesized on a solid support using standard light-directed methods (VLSIPS™ protocols). Two hexaethylene glycol (PEG) linkers were used to covalently attach the synthesized oligonucleotides to the derivatized glass surface. Synthesis of the first (inner) strand proceeded one nucleotide at a time using repeated cycles of photo-deprotection and chemical coupling of protected nucleotides. The nucleotides each had a protecting group on the base portion of the monomer as well as a photolabile MeNPoc protecting group on the 5' hydroxyl. Upon completion of the inner strand, another MeNPoc-protected PEG linker was covalently attached to the 5' end of the surface-bound oligonucleotide. After addition of the internal PEG linker, the PEG is photodeprotected, and the synthesis of the second strand proceeded in the normal fashion. Following the synthesis cycles, the DNA bases were deprotected using standard protocols. The sequence of the second (outer) strand, being complementary to that of the inner strand, provided molecules with short, hydrogen bonded, unimolecular double-stranded structure as a result of the presence of the internal flexible PEG linker.

An array of 16 different molecules were synthesized on a derivatized glass slide in order to determine whether short, unimolecular DNA structures could be formed on a surface and whether they could adopt structures that are recognized by proteins. Each of the 16 different molecular species occupies a different physical region on the glass surface so that there is a one-to-one correspondence between molecular identity and physical location. The molecules are of the form



where S is the solid surface having silyl groups, P is a PEG linker, A, C, G, and T are the DNA nucleotides, and F is a fluorescent tag. The DNA sequence is listed from the 3' to the 5' end (the 3' end of the DNA molecule is attached to the solid surface via a silyl group and 2 PEG linkers). The sixteen molecules synthesized on the solid support differed in the various permutations of A and T in the above formula.

Example 2

This example illustrates the ability of a library of surface-bound, unimolecular, double-stranded oligonucleotides to exist in duplex form and to be recognized and bound by a protein.

A library of 16 different members was prepared as described in Example 1. The 16 molecules all have the same composition (same number of As, Cs, Gs and Ts), but the order is different. Four of the molecules have an outer strand that is 100% complementary to the inner strand (these molecules will be referred to as DS, doublestranded, below). One of the four DS oligonucleotides has a sequence that is recognized by the restriction enzyme EcoRI. If the molecule can loop back and form a DNA duplex, it should be recognized and cut by the restriction enzyme, thereby releasing the fluorescent tag. Thus, the action of the enzyme

provided a functional test for DNA structure, and also served to demonstrate that these structures can be recognized at the surface by proteins. The remaining 12 molecules had outer strands that were not complementary to their inner strands (referred to as SS, single-stranded, below). Of these, three had an outer strand and three had an inner strand whose sequence was an EcoRI half-site (the sequence on one strand was correct for the enzyme, but the other half was not). The solid support with an array of molecules on the surface is referred to as a "chip" for the purposes of the following discussion. The presence of fluorescently labelled molecules on the chip was detected using confocal fluorescence microscopy. The action of various enzymes was determined by monitoring the change in the amount of fluorescence from the molecules on the chip surface (e.g. "reading" the chip) upon treatment with enzymes that can cut the DNA and release the fluorescent tag at the 5' end.

The three different enzymes used to characterize the structure of the molecules on the chip were:

- 1) Mung Bean Nuclease—sequence independent, single-strand specific DNA endonuclease;
- 2) DNase I—sequence independent, double-strand specific endonuclease;
- 3) EcoRI—restriction endonuclease that recognizes the sequence (5'-3')

GAATTC in double stranded DNA, and cuts between the G and the first A. Mung Bean Nuclease and EcoRI were obtained from New England Biolabs, and DNase I was obtained from Boehringer Mannheim. All enzymes were used at a concentration of 200 units per mL in the buffer recommended by the manufacturer. The enzymatic reactions were performed in a 1 mL flow cell at 22° C., and were typically allowed to proceed for 90 minutes.

Upon treatment of the chip with the enzyme EcoRI, the fluorescence signal in the DS EcoRI region and the 3 SS regions with the EcoRI half-site on the outer strand was reduced by about 10% of its initial value. This reduction was at least 5 times greater than for the other regions of the chip, indicating that the action of the enzyme is sequence specific on the chip. It was not possible to determine if the factor is greater than 5 in these preliminary experiments because of uncertainty in the constancy of the fluorescence background. However, because the purpose of these early experiments was to determine whether unimolecular double-stranded structures could be formed and whether they could be specifically recognized by proteins (and not to provide a quantitative measure of enzyme specificity), qualitative differences between the different synthesis regions were sufficient.

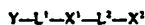
The reduction in signal in the 3 SS regions with the EcoRI half-site on the outer strand indicated either that the enzyme cuts single-stranded DNA with a particular sequence, or that these molecules formed a double-stranded structure that was recognized by the enzyme. The molecules on the chip surface were at a relatively high density, with an average spacing of approximately 100 angstroms. Thus, it was possible for the outer strand of one molecule to form a double-stranded structure with the outer strand of a neighboring molecule. In the case of the 3 SS regions with the EcoRI half-site on the outer strand, such a bimolecular double-stranded region would have the correct sequence and structure to be recognized by EcoRI. However, it would differ from the unimolecular double-stranded molecules in that the inner strand remains single-stranded and thus amenable to cleavage by a single-strand specific endonuclease such as Mung Bean Nuclease. Therefore, it was possible to distinguish unimolecular from bimolecular double-stranded

DNA molecules on the surface by their ability to be cut by single and double-strand specific endonucleases.

In order to remove all molecules that have single-stranded structures and to identify unimolecular double-stranded molecules, the chip was first exhaustively treated with Mung Bean Nuclease. The reduction in the fluorescence signal was greater by about a factor of 2 for the SS regions of the chip, including those with the EcoRI half-site on the outer strand that were cleaved by EcoRI, than for the 4 DS regions. Following Mung Bean Nuclease treatment, the chip was treated with either DNase I (which cuts all remaining double-stranded molecules) or EcoRI (which should cut only the remaining double-stranded molecules with the correct sequence). Upon treatment with DNase I, the fluorescence signal in the 4 DS regions was reduced by at least 5-fold more than the signal in the SS regions. Upon EcoRI treatment, the signal in the single DS region with the correct EcoRI sequence was reduced by at least a factor of 3 more than the signal in any other region on the chip. Taken together, these results indicated that the surface-bound molecules synthesized with two complementary strands separated by a flexible PEG linker form intramolecular double-stranded structures that were resistant to a single-strand specific endonuclease and were recognized by both a double-strand specific endonuclease, and a sequence-specific restriction enzyme.

What is claimed is:

1. A synthetic unimolecular, double-stranded oligonucleotide library comprising a plurality of different members, each member having the formula:



wherein,

Y is a solid support;

X¹ and X² are a pair of complementary oligonucleotides;

L¹ is a spacer;

L² is a linking group having sufficient length such that X¹ and X² form a double-stranded oligonucleotide.

2. A library in accordance with claim 1, wherein L² is a polyethylene glycol group.

3. A library in accordance with claim 1, wherein X¹ and X² are complementary oligonucleotides each comprising of from 6 to 30 nucleic acid monomers.

4. A library in accordance with claim 1, wherein said solid support is a silica support and L¹ comprises an aminoalkylsilane and from 1 to 4 hexaethyleneglycols.

5. A library in accordance with claim 1, wherein said solid support is a silica support, L¹ comprises an aminoalkylsilane and from 1 to 4 hexaethyleneglycols, L² is a polyethyleneglycol group and X¹ and X² are complementary oligonucleotides each comprising of from 6 to 30 nucleic acid monomers.

6. A synthetic unimolecular, double-stranded oligonucleotide library of claim 1, wherein a portion of said double-stranded oligonucleotides formed by X¹ and X² further comprise a loop.

G

This Page Blank (uspto)

United States Patent [19]

Fodor et al.

[11] Patent Number: 5,744,305
 [45] Date of Patent: *Apr. 28, 1998

[54] ARRAYS OF MATERIALS ATTACHED TO A SUBSTRATE

[75] Inventors: Stephen P.A. Fodor, Palo Alto; Lubert Stryer, Stanford; J. Leighton Read, Palo Alto, all of Calif.; Michael C. Pirrung, Durham, N.C.

[73] Assignee: Affymetrix, Inc., Santa Clara, Calif.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,445,934.

[21] Appl. No.: 466,632

[22] Filed: Jun. 6, 1995

Related U.S. Application Data

[60] Division of Ser. No. 390,272, Feb. 16, 1995, Pat. No. 5,489,678, and a continuation-in-part of Ser. No. 456,887, Jun. 1, 1995, which is a division of Ser. No. 954,646, Sep. 30, 1992, Pat. No. 5,445,934, which is a division of Ser. No. 850,356, Mar. 12, 1992, Pat. No. 5,405,783, which is a division of Ser. No. 492,462, Mar. 7, 1990, Pat. No. 5,143,854, which is a continuation-in-part of Ser. No. 362,901, Jun. 7, 1989, abandoned, said Ser. No. 390,272, is a continuation of Ser. No. 624,120, Dec. 6, 1990, abandoned, which is a continuation-in-part of Ser. No. 492,462, Mar. 7, 1990, Pat. No. 5,143,854, which is a continuation-in-part of Ser. No. 362,901, Jun. 7, 1989, abandoned.

[51] Int. Cl.⁶ C12Q 1/68; C07H 21/04; C07H 21/02

[52] U.S. Cl. 435/6; 435/7.92; 435/7.94; 435/7.95; 435/969; 435/973; 436/518; 436/527; 436/807; 436/809; 530/334; 536/24.3; 536/25.3; 536/25.32

[58] Field of Search 435/6, 7.92, 7.95, 435/7.94, 9.73, 969; 436/518, 527, 807, 809; 530/334; 536/24.3, 25.3, 25.32

[56] References Cited

U.S. PATENT DOCUMENTS

3,849,137 11/1974 Barzynski et al.
 4,269,933 5/1981 Pazos
 4,516,833 5/1985 Pasak

4,517,338 5/1985 Urdea et al.
 4,537,861 8/1985 Elings et al.
 4,562,157 12/1985 Lowe et al. 435/291
 4,631,211 12/1986 Houghton 428/35
 4,689,405 8/1987 Frank et al.
 4,704,353 11/1987 Humphries et al. 435/4
 4,713,326 12/1987 Dattagupta et al. 435/6
 4,728,591 3/1988 Clark et al.
 4,762,881 8/1988 Kauer 525/54.11

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

0 046 083 2/1982 European Pat. Off. B01F 17/00
 0 103 197 3/1984 European Pat. Off. C07C 79/46
 0 228 310 10/1988 European Pat. Off.
 0 328 256 A1 1/1989 European Pat. Off. B01J 20/32
 0 392 546 10/1990 European Pat. Off. C12Q 1/68

(List continued on next page.)

OTHER PUBLICATIONS

Barinaga, "Will 'DNA Chip' Speed Genome Initiative?" *Science*, 253:1489 (1991).
 BioRad Catalogue M 1987, p. 182.
 Geyson et al., "Strategies for epitope analysis using peptide synthesis," *J. Immunol. Methods*, 102:259-274 (1987).

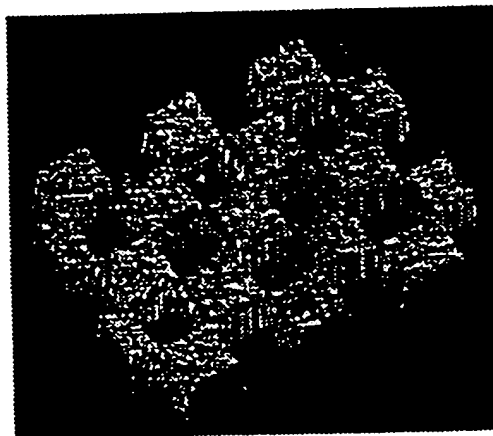
(List continued on next page.)

Primary Examiner—Stephanie W. Zitomer
 Assistant Examiner—Paul B. Tran
 Attorney, Agent, or Firm—Vern Norvick; Nancy J. DeSantis; Joseph Liebeschuetz

[57] ABSTRACT

A synthetic strategy for the creation of large scale chemical diversity. Solid-phase chemistry, photolabile protecting groups, and photolithography are used to achieve light-directed spatially-addressable parallel chemical synthesis. Binary masking techniques are utilized in one embodiment. A reactor system, photoremovable protective groups, and improved data collection and handling techniques are also disclosed. A technique for screening linker molecules is also provided.

26 Claims, 17 Drawing Sheets



U.S. PATENT DOCUMENTS

4,811,062	3/1989	Taba et al.	
4,833,092	5/1989	Geysen	436/501
4,846,552	7/1989	Veldkamp et al.	350/162.2
4,886,741	12/1989	Schwartz	
4,888,278	12/1989	Singer et al.	
4,923,901	5/1990	Koester et al.	
4,946,942	8/1990	Füller et al.	530/335
4,973,493	11/1990	Gaire	
4,981,985	1/1991	Kaplan et al.	
4,984,100	1/1991	Takayama et al.	360/49
5,079,600	1/1992	Schnur et al.	
5,143,854	9/1992	Pirrung et al.	
5,202,231	4/1993	Dzmanac et al.	
5,252,743	10/1993	Barrett et al.	
5,258,506	11/1993	Urdea et al.	
5,445,934	8/1995	Fodor et al.	435/6

FOREIGN PATENT DOCUMENTS

1-233 447	9/1989	Japan	
2 196 476	2/1990	United Kingdom	H01L 21/46
WO 84/03564	9/1984	WIPO	G01N 33/54
WO 86/06487	11/1986	WIPO	G01N 33/53
WO 89/10977	11/1989	WIPO	C12Q 1/68
WO 89/11548	11/1989	WIPO	C12Q 1/68
WO 90/03382	4/1990	WIPO	C07H 21/00
WO 90/04652	5/1990	WIPO	C12Q 1/68
WO 90/15070	12/1990	WIPO	C07K 1/104
WO 91/07087	5/1991	WIPO	A01N 1/02

OTHER PUBLICATIONS

Haynes & Higgins (eds.), *Nucleic Acid Hybridization: A Practical Approach*. IRL Press, Oxford, England, pp. 126-128 (1985).

Mirzabekov, "DNA sequencing by hybridization—a megasequencing method and a diagnostic tool?" *TIBTECH*, 12:27-32 (1994).

Southern et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of oligonucleotides: Evaluation Using Experimental Models." *Genomics*, 13:1008-1017 (1992).

"A Sequencing Reality Check", *Research News [Science]* (1988) 242:1245.

"Affymax Raises \$25 Million to Develop High Speed Drug Discovery System" *Biotechnology News*, vol. 10, No. 3, Feb. 1, 1990, pp. 7-8.

Adams et al., "Photolabile chelators that 'cage' calcium with improved speed of release and pre-photolysis affinity," *J. General Physiology* (Dec. 1986).

Adams et al., "Biologically useful chelators that take up Ca^{2+} upon illumination," *J. Am. Chem. Soc.* 111:7957-7968 (1989).

Amit et al., "Photosensitive protecting groups of amino sugars and their use in glycoside synthesis. 2-Nitrobenzyl-oxycarbonylamino and 6-Nitroveratryloxy-carbonylamino derivatives," *J. Org. Chem.* (1974) 39:192-196.

Amit et al., "Photosensitive protecting groups—A review," *Israel J. of Chem.* 12(1-2):103-113 (1974).

Bains et al., "A novel method for nucleic acid sequence determination," *J. Theor. Biol.* (1988) 135:303-307.

Baldwin et al., "New photolabile phosphate protecting groups," *Tetrahedron* 46(19):6879-6884 (1990).

Bartrop et al., "Photosensitive protective groups," *Chemical Communications*, p. 822 (Nov. 22, 1966).

Cameron et al., "Photogeneration of organic bases from o-nitrobenzyl-derived carbamates," *J. Am. Chem. Soc.* (1991) 113, 4303-4313.

Craig et al., "Ordering of cosmid clones covering the herpes simplex virus type 1 (HSV-1) genome: a test case for fingerprinting by hybridisation," *Nucl. Acids Res.* (1990) 18:2653-2660.

Cummings et al., "Photoactivable fluorophores. 1. Synthesis and photoactivation of o-nitrobenzyl-quenched fluorescent carbamates," *Tetrahedron Letters* (1988) 29:65-68.

Dzmanac et al., "Sequencing of megabase plus DNA by hybridization: theory of the method," *Genomics* (1989) 4:114-128.

Dulcey et al., "Deep UV photochemistry of chemisorbed monolayers: patterned coplanar molecular assemblies," *Science* (1991) 252:551-554.

Flanders et al., "A new interferometric alignment technique," *App. Phys. Lett.* (1977) 31:426-428.

Fodor et al., "Light-directed Spatially-addressable Parallel Chemical Synthesis," *Science* 251:767-773 (1991).

Furka et al., "General method for rapid synthesis of multi-component peptide mixtures," *Int. J. Peptide Protein Res.* (1991) 37:487-493.

Furka, et al., "Cornucopia of peptides by synthesis," 14th Int'l Congress of Biochem., Abstract No. FR:013, Prague, Czechoslovakia, Jul. 10-15, 1988.

Furka et al., "More peptides by less labour," Xth Int'l Symposium on Medicinal Chemistry, (Abstract No. 288) Budapest, Hungary, Aug. 15-19, 1988.

Gurney et al., Activation of a potassium current by rapid photochemically generated step increases of intracellular calcium in rat sympathetic neurons. *PNAS USA* 84:3496-3500 (May 1987).

Haridasan et al., "Peptide synthesis using photolytically cleavable 2-nitrobenzylloxycarbonyl protecting group," *Proc. Indian Natl. Sci. Acad. Part A* (1987) 53:717-728.

Iwamura et al., "1-pyrenylmethyl esters, photolabile protecting groups for carboxylic acids," *Tetrahedron Letters* (1987) 28:679-682.

Iwamura et al., "1-x-Diazobenzyl pyrene: A reagent for photolabile and fluorescent protection of carboxyl groups of amino acids and peptides," *Chemical Abstracts*, vol. 114(23) (1991).

Iwamura et al., "1-(α -Diazobenzyl) pyrene: A reagent for photolabile and fluorescent protection of carboxyl groups of amino acids and peptides," (1991) *Synlett* 35-36.

Kaplan et al., "Photolabile chelators for the rapid photorelease of divalent cations," *PNAS USA* 85:6571-6575 (Sep. 1988).

Khrapko et al., "An oligonucleotide hybridization approach to DNA sequencing," *FEBS. Lett.* (1989) 256:118-122.

Kleinfeld et al., "Controlled outgrowth of dissociated neurons on patterned substrates," *J. of Neuroscience* 8(11):4098-4120 (Nov. 1988).

Krile et al., "Multiplex holography with chip-modulated binary phase-coded reference-beam masks," *Applied Optics* (1979) 18:52-56.

Lam et al., "A new type of synthetic peptide library for identifying ligand-binding activity," *Nature* (1991) 354:82-86.

Logue et al., "General approaches to mask design for binary optics," *SPIE* (1989) 1052:19-24.

- Lysov et al., "A new method for determining the DNA nucleotide sequence by hybridization with oligonucleotides," *Doklady Akademii Nauk SSR* (1988) 303:1508-1511.
- McCray et al., "Properties and uses of photoreactive caged compounds," *Ann. Rev. Biophys. and Biophys. Chem.* (1989) 18:239-270.
- McGillis, "Lithography," *VLSI Technology*, S. Sze, ed., McGraw-Hill Book Company, 1983, pp. 267-301.
- Ohtsuka et al., "Studies on transfer ribonucleic acids and related compounds. IX(1) Ribooligonucleotide synthesis using a photosensitive o-nitrobenzyl protection at the 2'-hydroxyl group," *Nucleic Acids Research* (1974) 1:1351-1357.
- Patchornik et al., "Photosensitive protecting groups," *J. Am. Chem. Soc.* (1970) 92:6333-6335.
- Patent Abstracts of Japan from the EPO, Abst. vol. 13:557, pub. date 12-28-89 abstracting Japanese Patent 01-233 447.
- Pillai et al., "3-nitro-4-aminomethyl-benzoyl derivative von poly-ethylenglykolen: eine neue klasse von photosensitiven loslichen polymeren tragern zur synthese von c-terminalen peptidamiden," *Tetrahedron Letters* (1979) No. 36, pp. 3409-3412.
- Pillai et al., "Photoremovable protecting groups in organic synthesis," *Synthesis* pp. 1-26 (Jan. 1980).
- Poustka et al., "Molecular approaches to mammalian genetics," *CSH Symp. Quant. Biol.* (1986) 51:131-139.
- Reichmanis et al., "o-nitrobenzyl photochemistry: Solution vs. solid-state behavior," *J. Polymer Sc. Polymer Chem. Ed.* 23:1-8 (1985).
- Robertson et al., "A general and efficient route for chemical aminoacylation of transfer RNAs," *Chemical Abstracts*, vol. 114, No. 15 (1991).
- Robertson et al., "A general and efficient route for chemical aminoacylation of transfer RNAs," (1991) *J. Am. chem. Soc.* 113:2722-2729.
- Schuup et al., "Mechanistic studies of the photorearrangement of o-nitrobenzyl esters," *J. Photochem.* 36:85-97 (1987).
- Shin et al., "Dehydrooligopeptides. XI. Facile syntheses of various kinds of dehydrodi- and tripeptides, and dehydroenkephalins containing Tyr residue by using N-carboxydehydrotyrosine anhydride," (1989) *Bull. Chem. Soc. Jpn.* 62:1127-1135.
- Shin et al., "Dehydrooligopeptides. XI. Facile synthesis of various kinds of dehydrodi- and tripeptides, and dehydroenkephalins containing Tyr residue by using N-carboxydehydrotyrosine anhydride," *Chemical Abstracts*, 112(11) 1990).
- Tsien et al., "Control of cytoplasmic calcium with photolabile tetracarboxylate 2-nitrobenzylhydrol chelators," *Biophys. J.* 50:843-853 (Nov. 1986).
- Veldkamp, "Binary optics: the optics technology of the 1990s," *CLEO 90*, May 21, 1990, Paper No. CMG6.
- Walker et al., "Photolabile protecting groups for an acetyl-choline receptor ligand. Synthesis and photochemistry of a new class of o-nitrobenzyl derivatives and their effects on receptor function," *Biochemistry* 25:1799-1805 (1986).
- Zehavi et al., "Light-sensitive glycosides. I. 6-nitroveratryl β -D-glucopyranoside and 2-nitrobenzyl β -D-glucopyranoside," *J. Org. Chem.* (1972) 37:2281-2285.
- Wilcox et al., "Synthesis of photolabile 'precursors' of amino acid neurotransmitters," *J. Org. Chem.* 55:1585-1589 (1990).

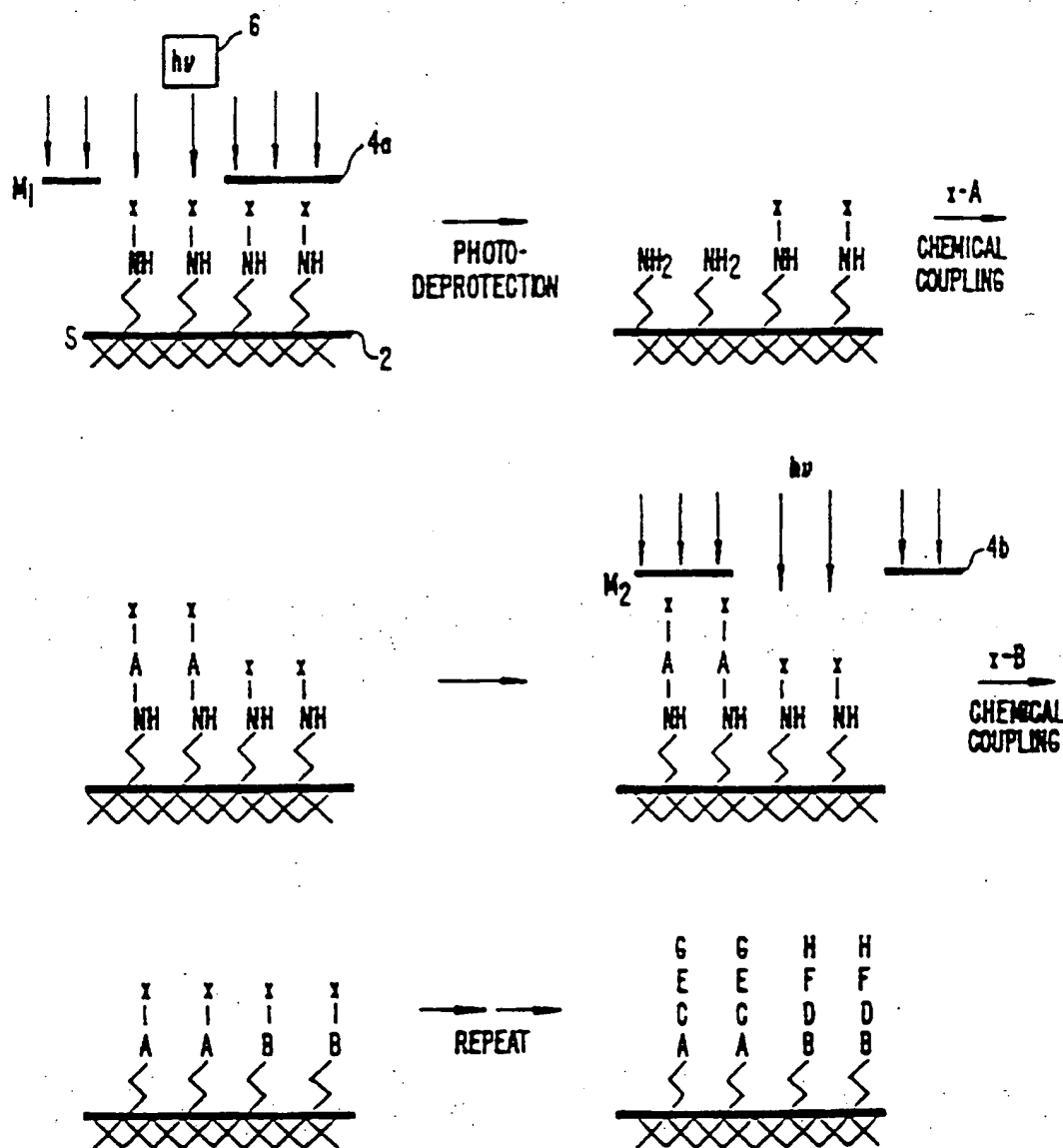


FIG. 1.

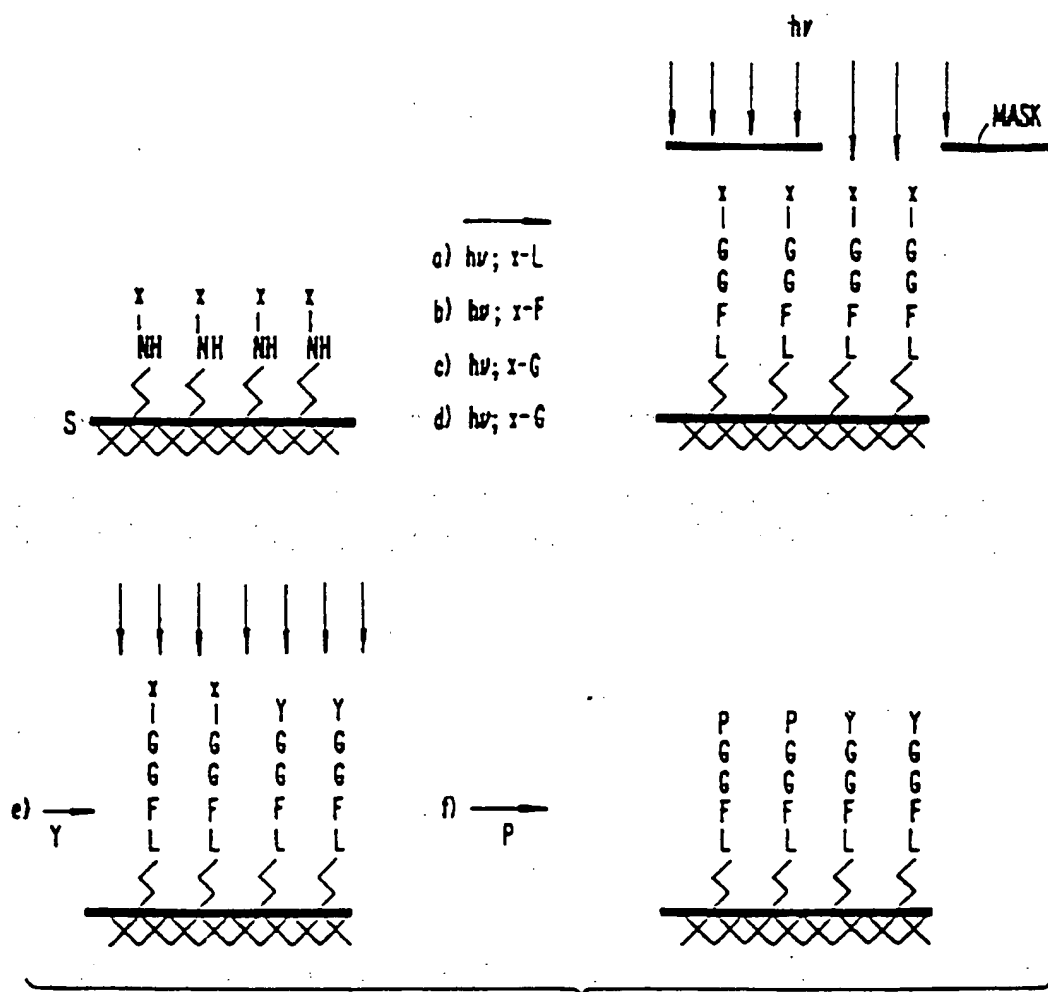


FIG. 2.

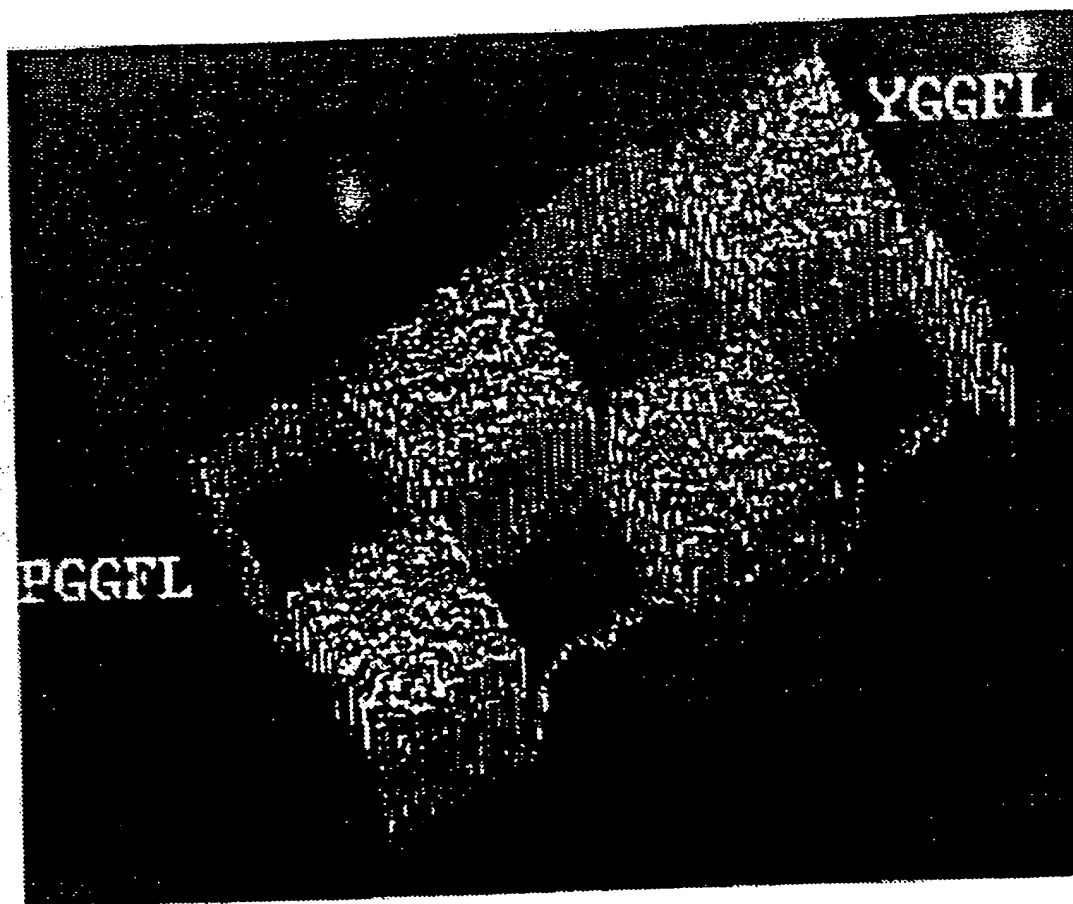


FIG. 3.

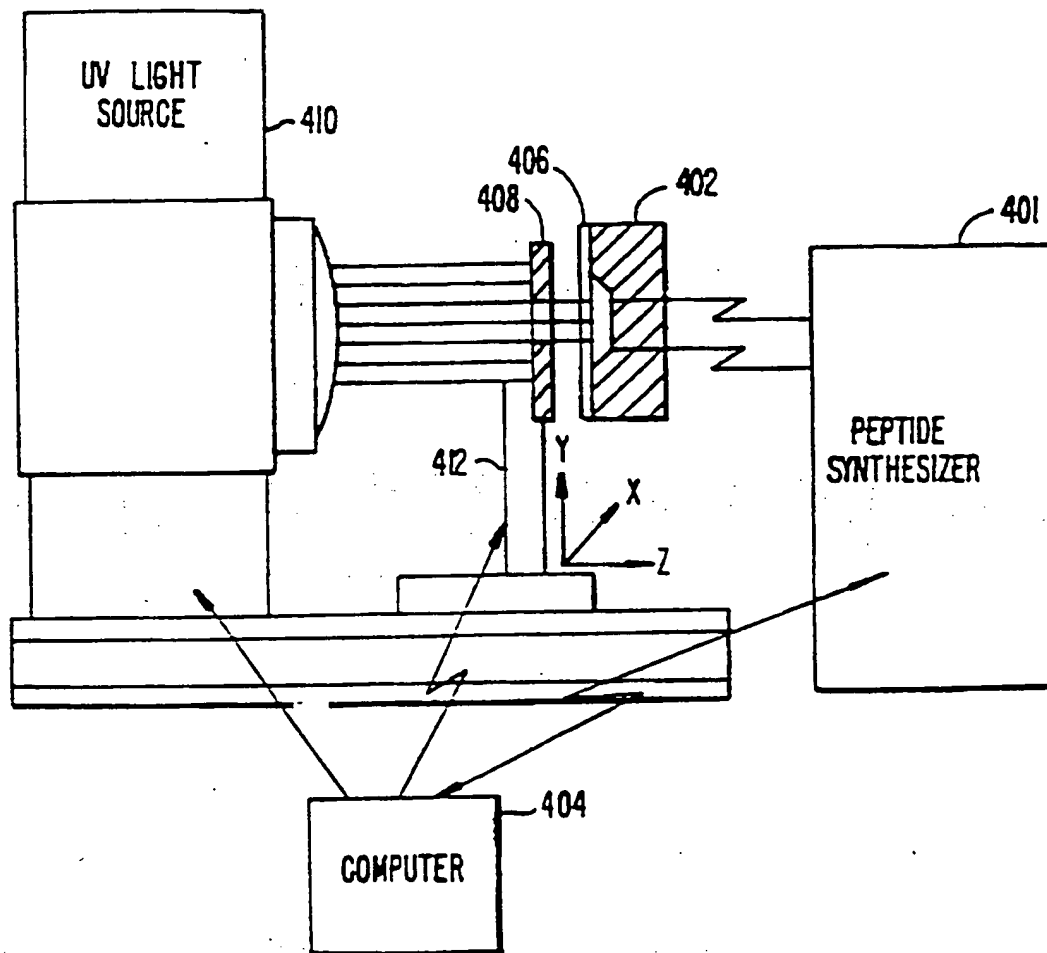


FIG. 4.

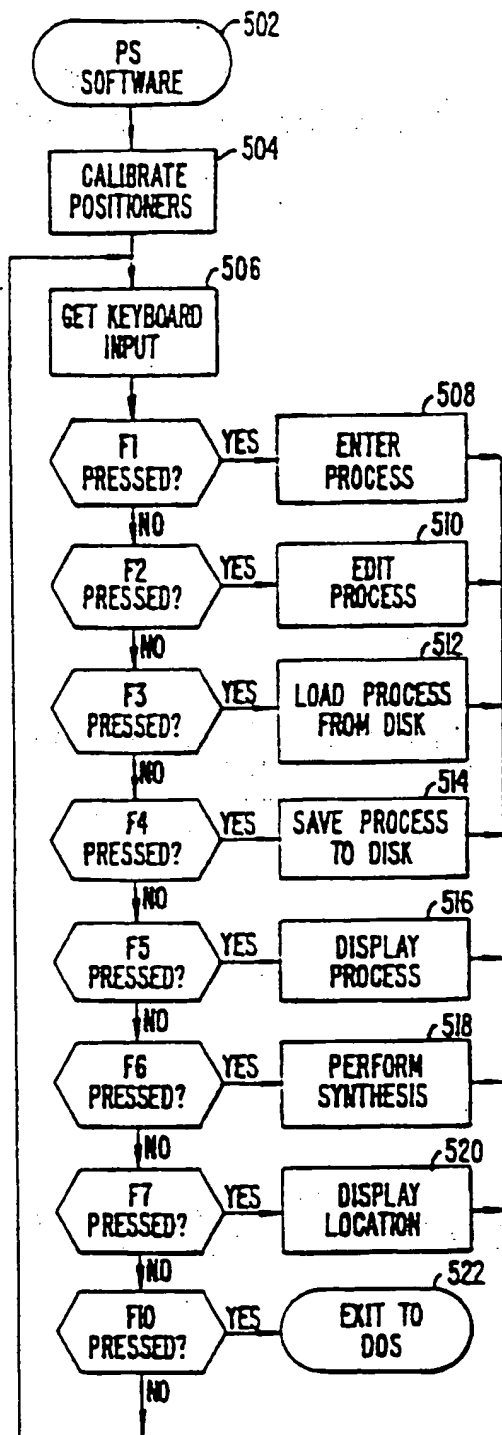


FIG. 5A.

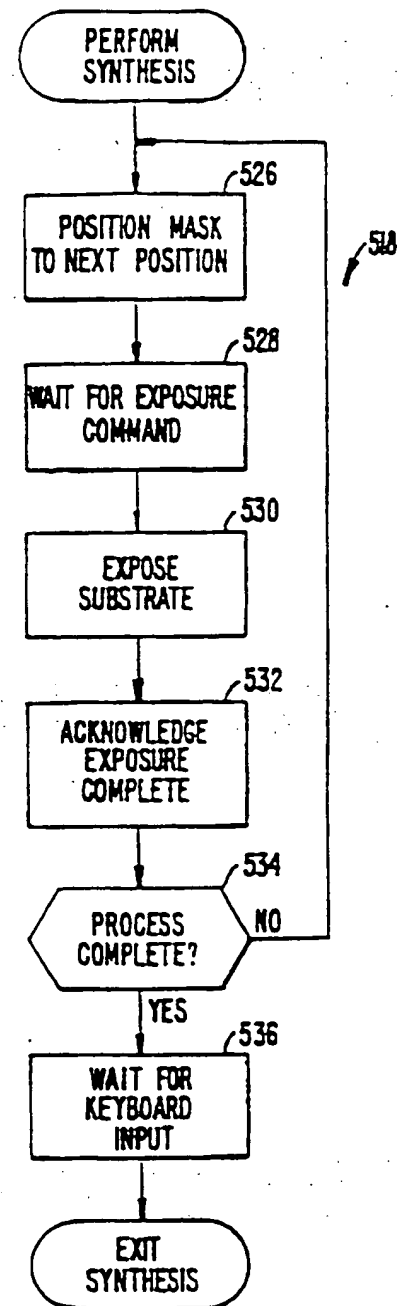


FIG. 5B.

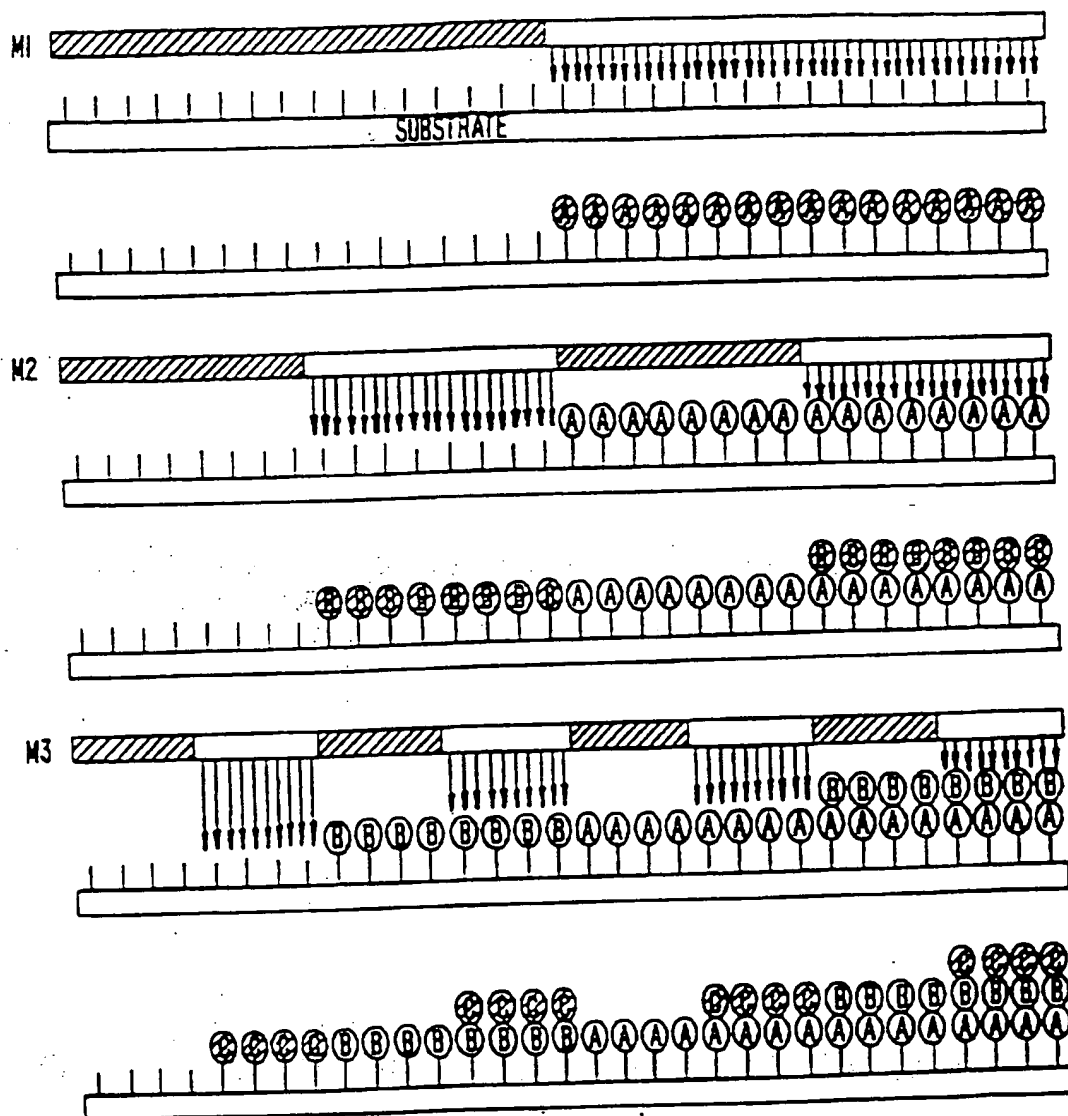


FIG. 6A.

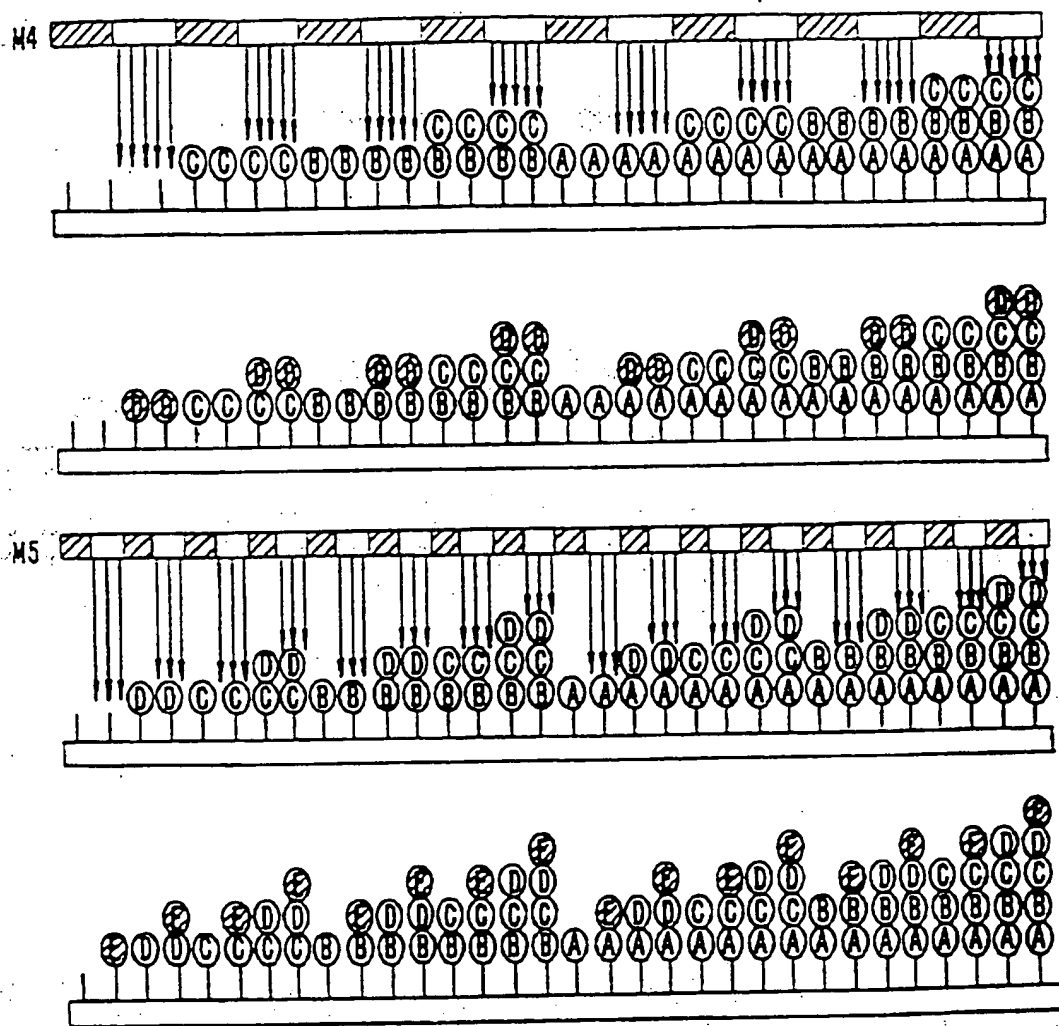


FIG. 6B.

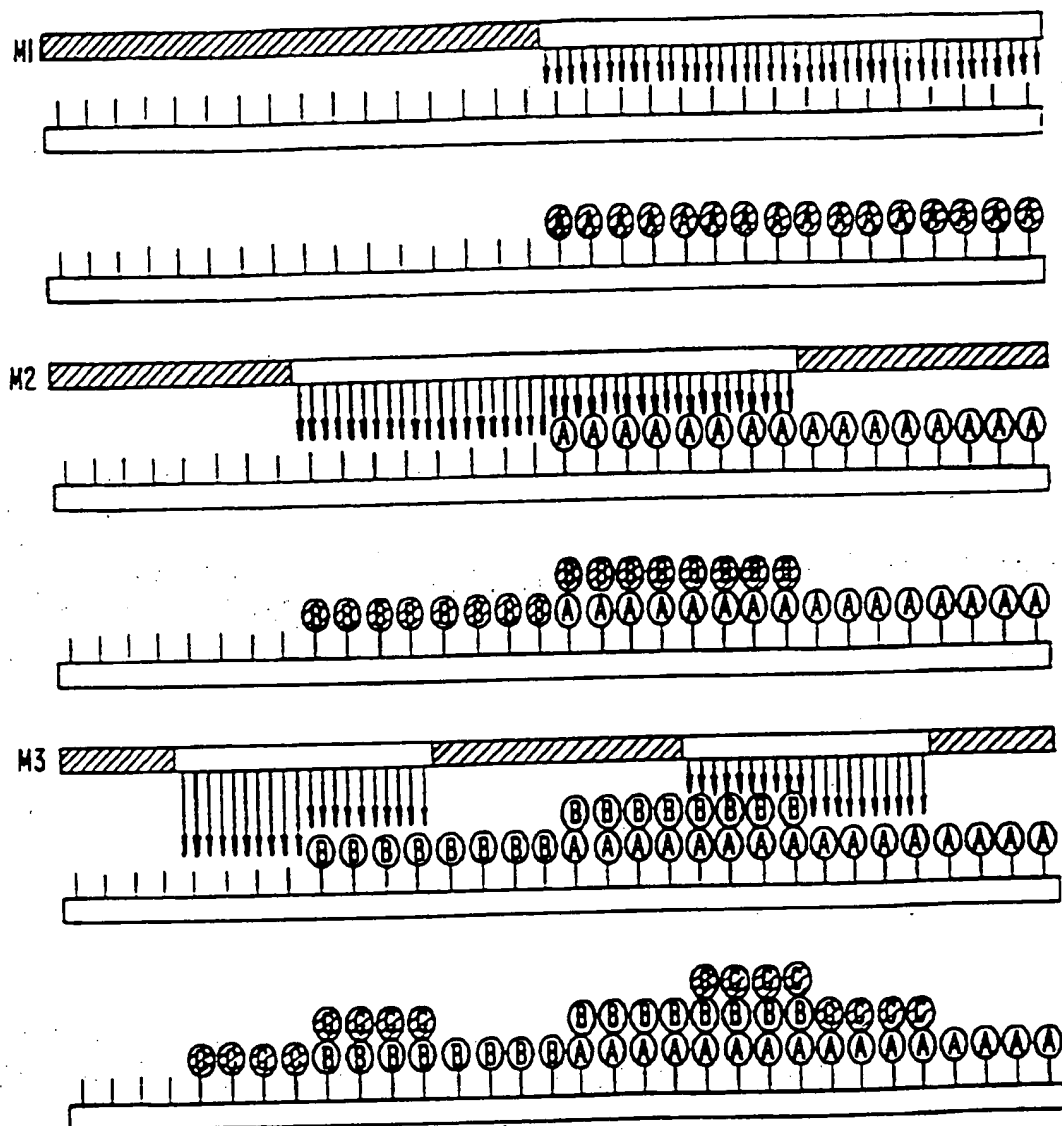


FIG. 7A.

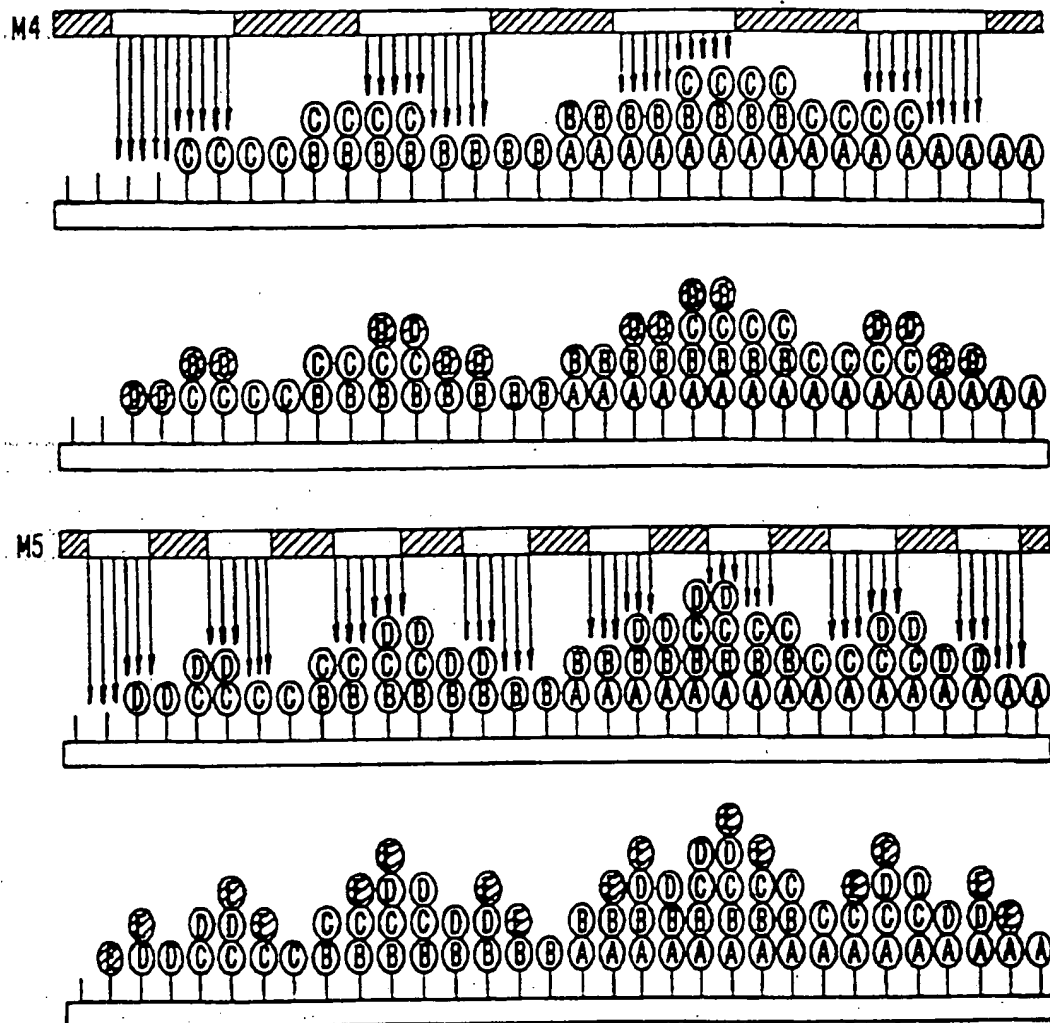


FIG. 7B.

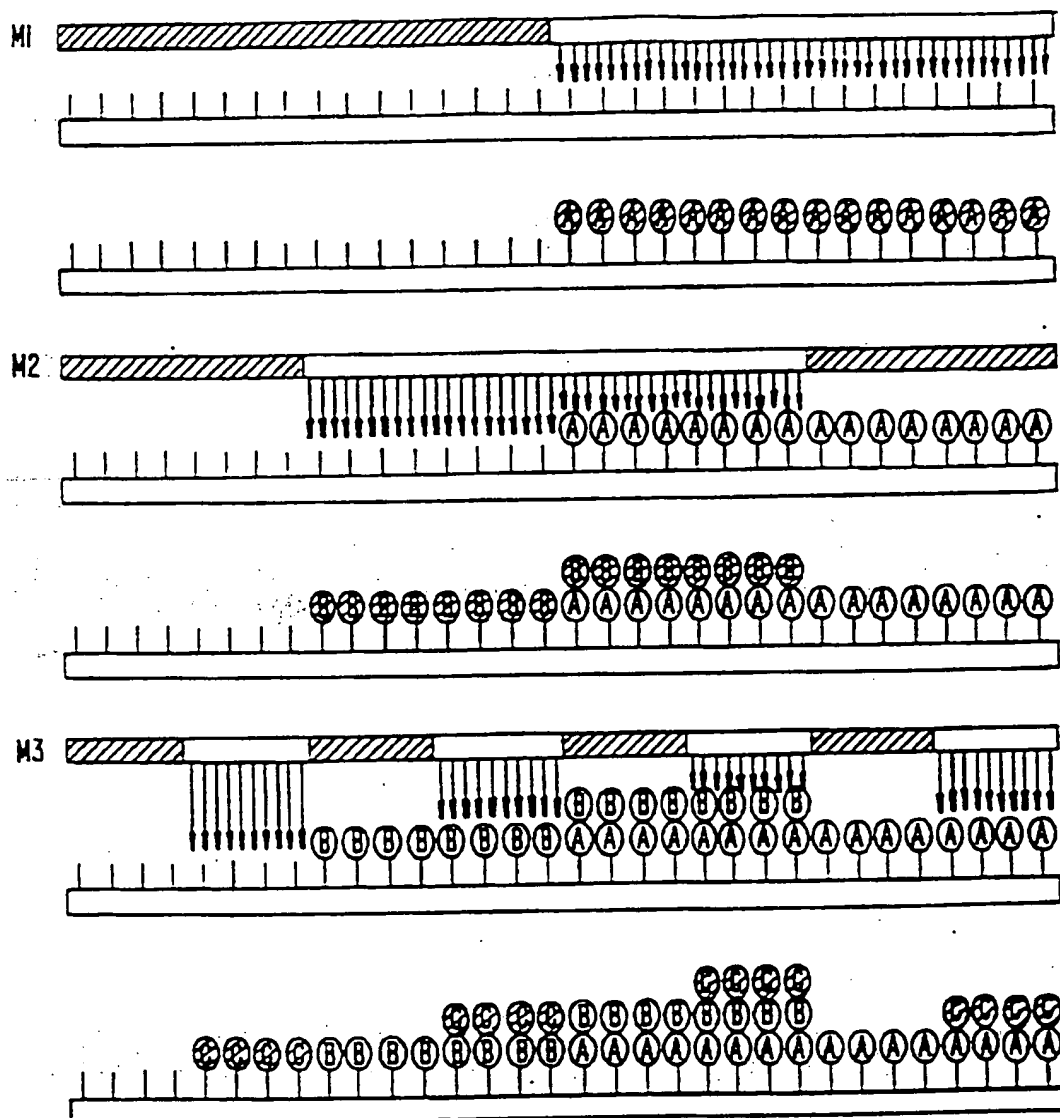


FIG. 8A.

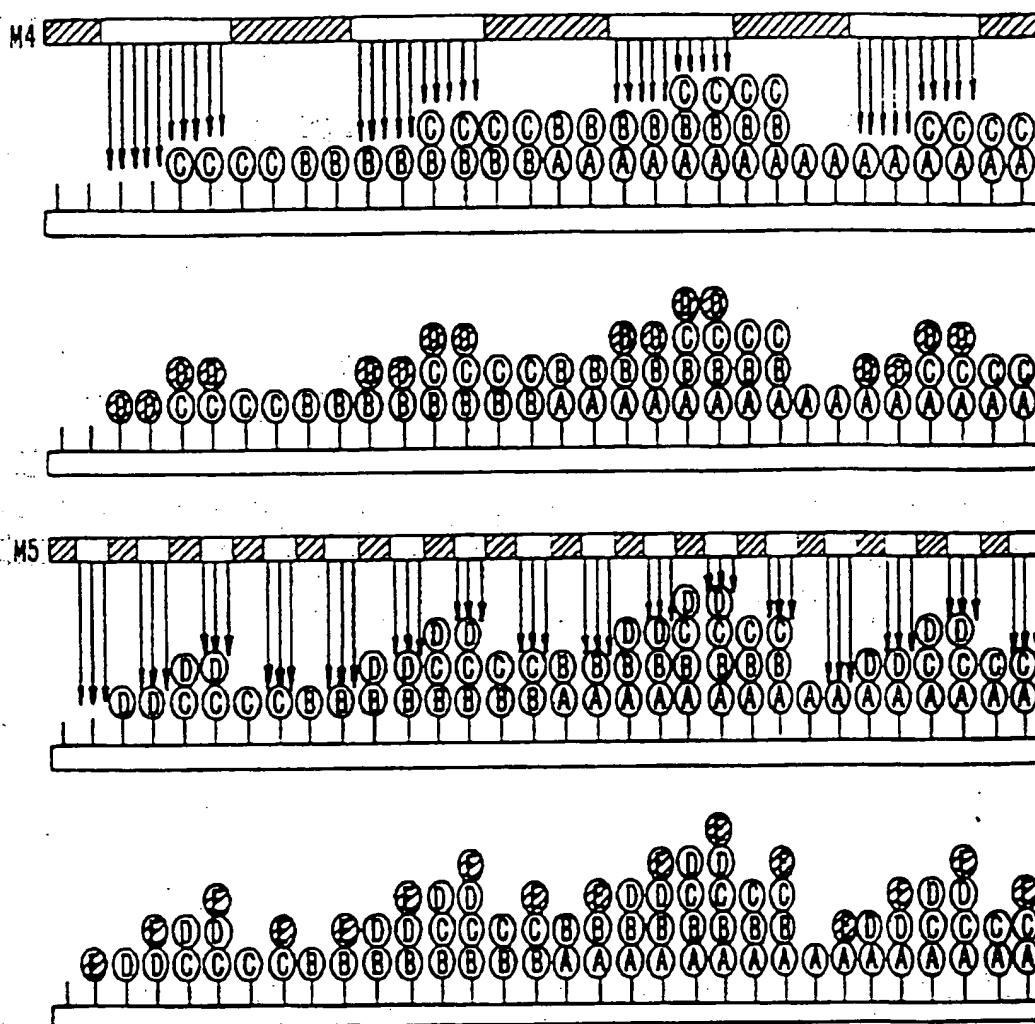


FIG. 8B.

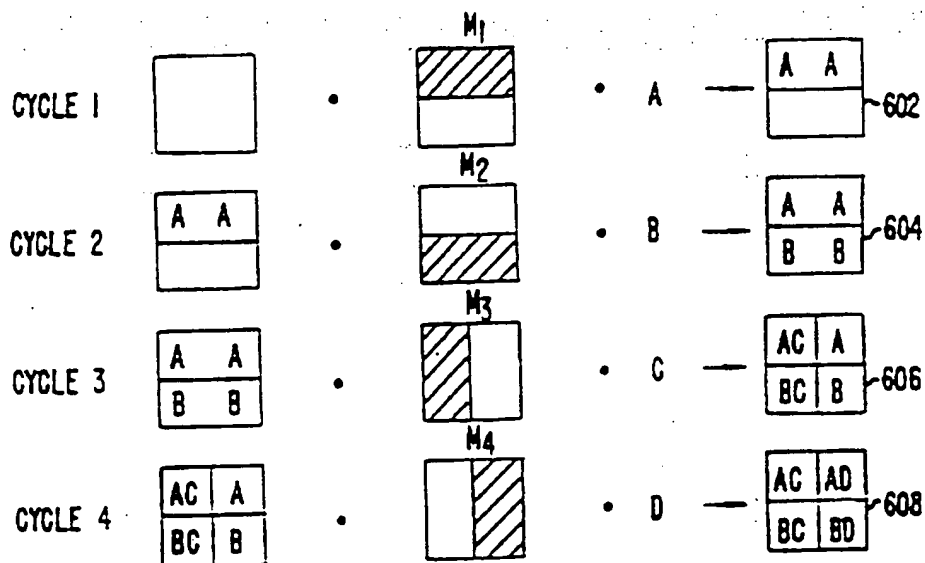


FIG. 9A.

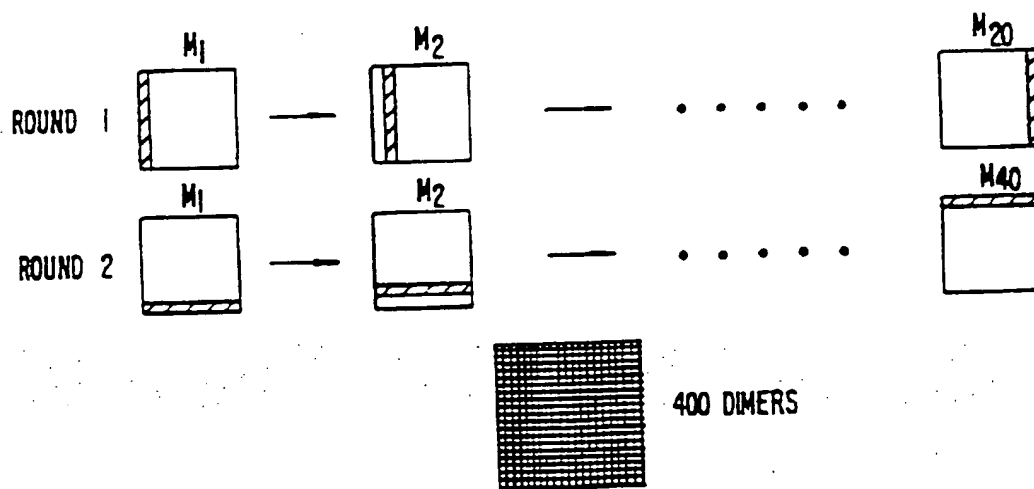


FIG. 9B.

(x, y)

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f
2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
4			A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
5	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
6			T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
7									F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
8																	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
9									S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10																	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F

FIG. 10.

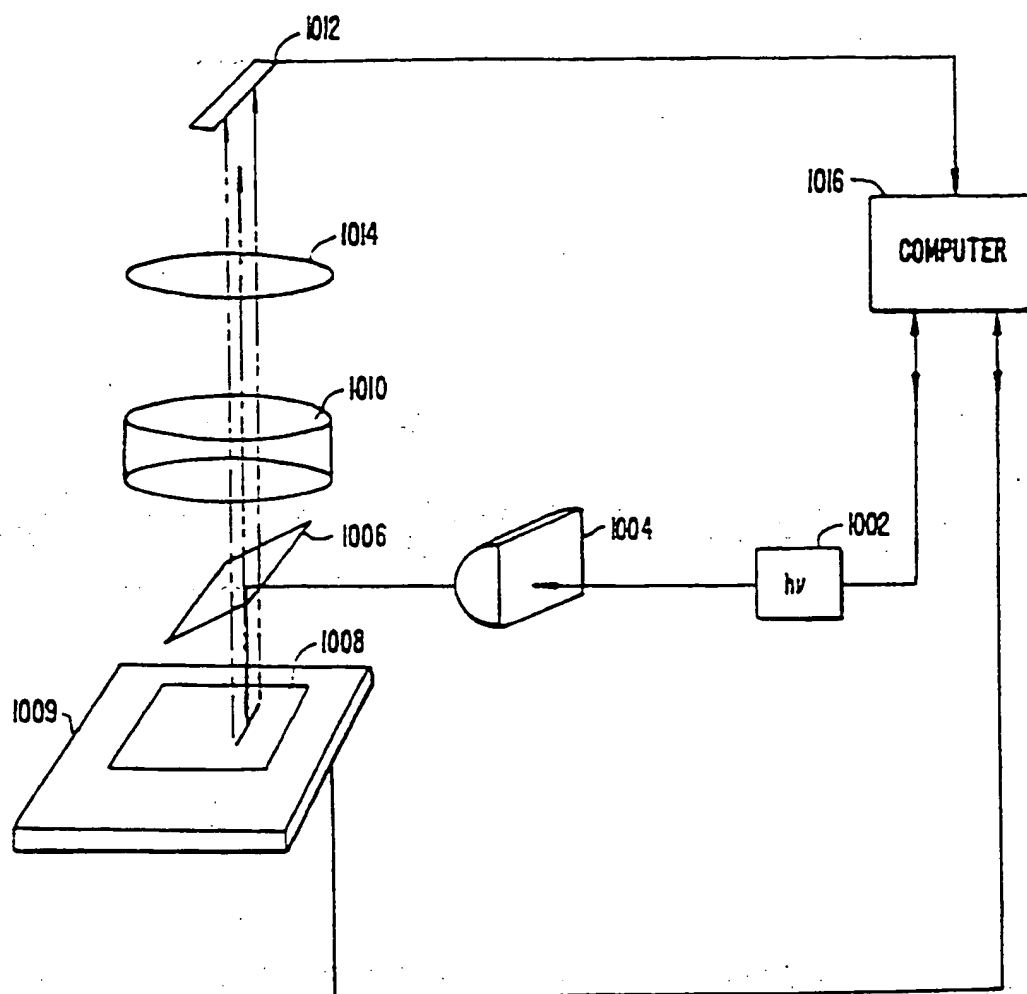


FIG. II.

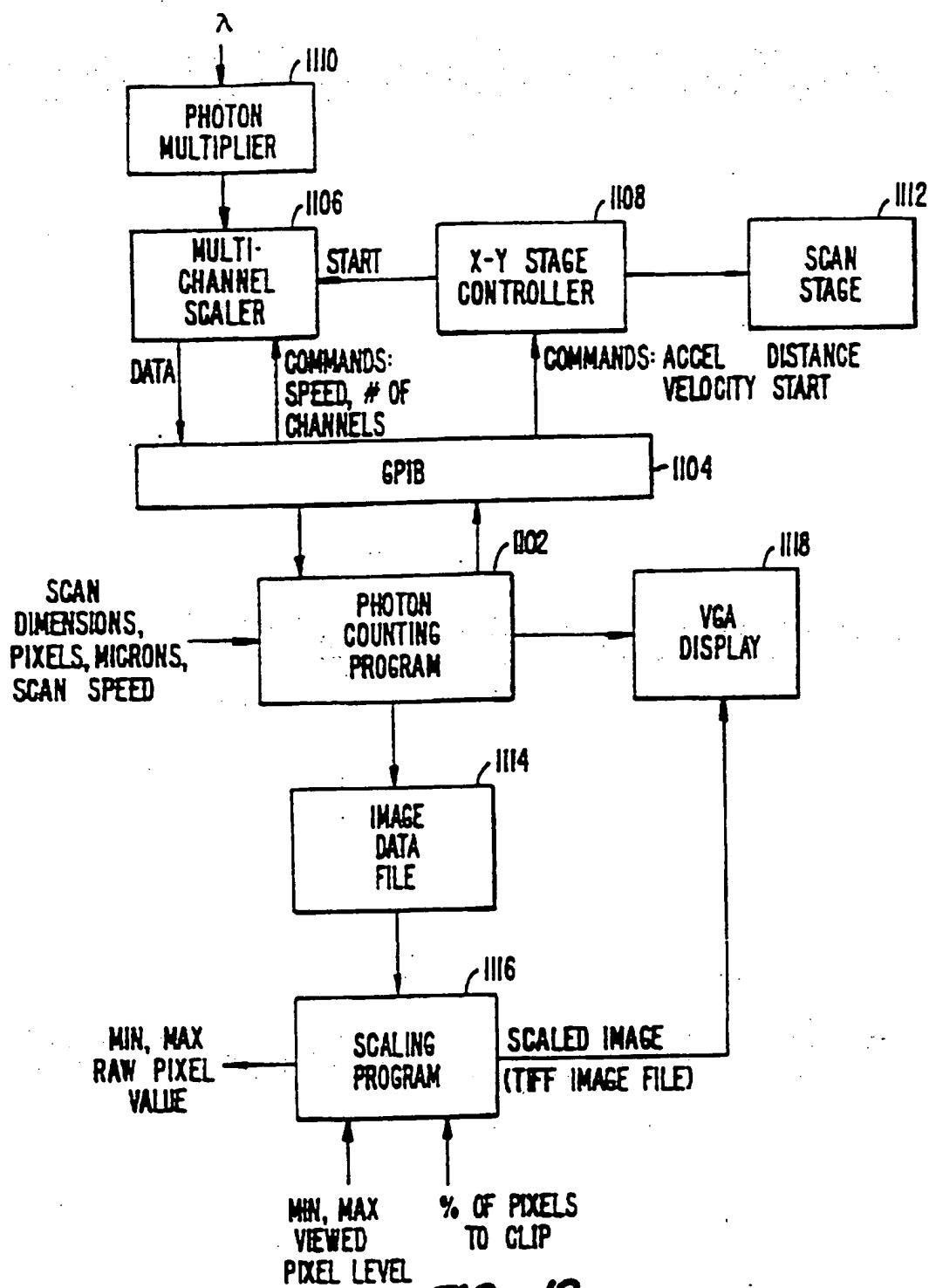


FIG. 12.

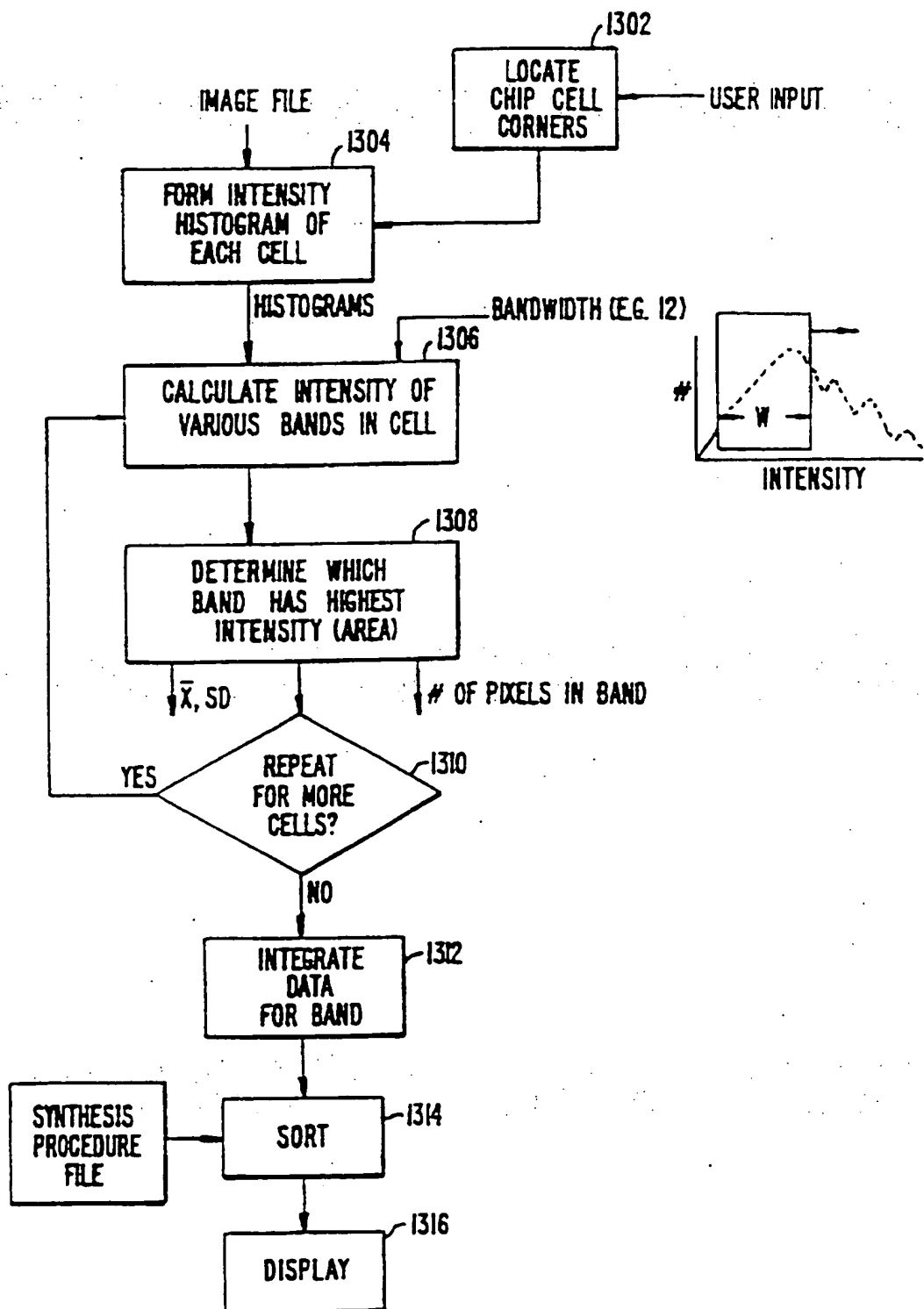


FIG. 13.

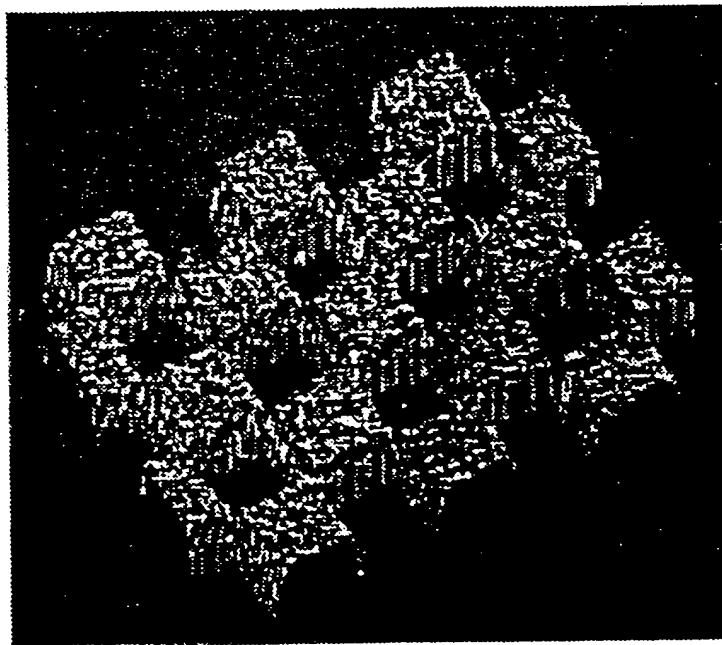


FIG. 14.

ARRAYS OF MATERIALS ATTACHED TO A SUBSTRATE

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a division of U.S. patent application Ser. No. 08/390,272, filed Feb. 16, 1995, now U.S. Pat. No. 5,489,678, which is a continuation of U.S. patent application Ser. No. 07/624,120, filed Dec. 6, 1990, now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 07/492,462, filed Mar. 7, 1990, now U.S. Pat. No. 5,143,854, which is a continuation-in-part of U.S. patent application Ser. No. 07/362,901, filed Jun. 7, 1989, now abandoned, and hereby incorporated herein by reference for all purposes. This application is also a continuation-in-part of U.S. patent application Ser. No. 08/456,887, filed Jun. 1, 1995, which is a division of U.S. patent application Ser. No. 07/954,646, filed Sep. 30, 1992, now U.S. Pat. No. 5,445,934, which is a division of U.S. patent application Ser. No. 07/850,356, filed Mar. 12, 1992, now U.S. Pat. No. 5,405,783, which is a division of U.S. patent application Ser. No. 07/492,462, filed Mar. 7, 1990, now U.S. Pat. Ser. No. 5,143,854, which is a continuation-in-part of U.S. patent application Ser. No. 07/362,901 filed Jun. 7, 1989, now abandoned.

This application is also related to U.S. patent application Ser. No. 08/670,118 filed Jun. 25, 1996, which is a division of U.S. patent application Ser. No. 08/168,104, filed Dec. 15, 1993, which is a continuation of U.S. patent application Ser. No. 07/624,114, filed Dec. 6, 1990, now abandoned, and U.S. patent application Ser. No. 07/626,730, filed Dec. 6, 1990, now U.S. Pat. No. 5,547,839, and also incorporated herein by reference for all purposes.

COPYRIGHT NOTICE

A portion of the disclosure of this patent document contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the Patent and Trademark Office patent file or records, but otherwise reserves all copyright rights whatsoever.

BACKGROUND OF THE INVENTION

The present invention relates to the field of polymer synthesis. More specifically, the invention provides a reactor system, a masking strategy, photoremovable protective groups, data collection and processing techniques, and applications for light directed synthesis of diverse polymer sequences on substrates.

SUMMARY OF THE INVENTION

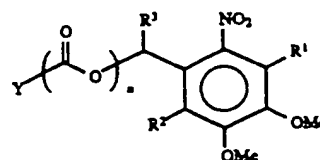
Methods, apparatus, and compositions for synthesis and use of diverse polymer sequences on a substrate are disclosed, as well as applications thereof.

According to one aspect of the invention, an improved reactor system for synthesis of diverse polymer sequences on a substrate is provided. According to this embodiment the invention provides for a reactor for contacting reaction fluids to a substrate; a system for delivering selected reaction fluids to the reactor; a translation stage for moving a mask or substrate from at least a first relative location relative to a second relative location; a light for illuminating the substrate through a mask at selected times; and an appropriately programmed digital computer for selectively directing a

flow of fluids from the reactor system, selectively activating the translation stage, and selectively illuminating the substrate so as to form a plurality of diverse polymer sequences on the substrate at predetermined locations.

The invention also provides a technique for selection of linker molecules in a very large scale immobilized polymer synthesis (VLSIPS™) method. According to this aspect of the invention, the invention provides a method of screening a plurality of linker polymers for use in binding affinity studies. The invention includes the steps of forming a plurality of linker polymers on a substrate in selected regions, the linker polymers formed by the steps of recursively: on a surface of a substrate, irradiating a portion of the selected regions to remove a protective group, and contacting the surface with a monomer; contacting the plurality of linker polymers with a ligand; and contacting the ligand with a labeled receptor.

According to another aspect of the invention, improved photoremovable protective groups are provided. According to this aspect of the invention a compound having the formula:

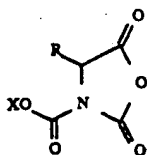


wherein $n=0$ or 1 ; Y is selected from the group consisting of an oxygen of the carboxyl group of a natural or unnatural amino acid, an amino group of a natural or unnatural amino acid, or the C-5' oxygen group of a natural or unnatural deoxyribonucleic or ribonucleic acid; R^1 and R^2 independently are a hydrogen atom, a lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido, sulfido, or phosphido group; and R^3 is a alkoxy, alkyl, aryl, hydrogen, or alkenyl group is provided.

The invention also provides improved masking techniques for the VLSIPS™ methodology. According to one aspect of the masking technique, the invention provides an ordered method for forming a plurality of polymer sequences by sequential addition of reagents comprising the step of serially protecting and deprotecting portions of the plurality of polymer sequences for addition of other portions of the polymer sequences using a binary synthesis strategy.

Improved data collection equipment and techniques are also provided. According to one embodiment, the instrumentation provides a system for determining affinity of a receptor to a ligand comprising: means for applying light to a surface of a substrate, the substrate comprising a plurality of ligands at predetermined locations, the means for providing simultaneous illumination at a plurality of the predetermined locations; and an array of detectors for detecting light fluoresced at the plurality of predetermined locations. The invention further provides for improved data analysis techniques including the steps of exposing fluorescently labelled receptors to a substrate, the substrate comprising a plurality of ligands in regions at known locations; at a plurality of data collection points within each of the regions, determining an amount of light fluoresced from the data collection points; removing the data collection points deviating from a predetermined statistical distribution; and determining a relative binding affinity of the receptor to remaining data collection points.

Protected amino acid N-carboxy anhydrides for use in polymer synthesis are also disclosed. According to this aspect, the invention provides a compound having the formula:



where R is a side chain of a natural or unnatural amino acid and X is a photoremovable protecting group.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically illustrates light-directed spatially-addressable parallel chemical synthesis;

FIG. 2 schematically illustrates one example of light-directed peptide synthesis;

FIG. 3 is a three-dimensional representation of a portion of the checkerboard array of YGGFL and PGGFL;

FIG. 4 schematically illustrates an automated system for synthesizing diverse polymer sequences;

FIGS. 5a and 5b illustrate operation of a program for polymer synthesis;

FIGS. 6a and 6b are a schematic illustration of a "pure" binary masking strategy;

FIGS. 7a and 7b are a schematic illustration of a gray code binary masking strategy;

FIGS. 8a and 8b are a schematic illustration of a modified gray code binary masking strategy;

FIG. 9a schematically illustrates a masking scheme for a four step synthesis;

FIG. 9b schematically illustrates synthesis of all 400 peptide dimers;

FIG. 10 is a coordinate map for the ten-step binary synthesis;

FIG. 11 schematically illustrates a data collection system;

FIG. 12 is a block diagram illustrating the architecture of the data collection system;

FIG. 13 is a flow chart illustrating operation of software for the data collection/analysis system; and

FIG. 14 illustrates a three-dimensional plot of intensity versus position for light directed synthesis of a dinucleotide.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS

I. Definitions

II. General

Deprotection and Addition

1. Example

2. Example

B. Antibody recognition

1. Example

III. Synthesis

A. Reactor System

B. Binary Synthesis Strategy

1. Example

2. Example

3. Example

4. Example

5. Example

6. Example

C. Linker Selection

D. Protecting Groups

1. Use of Photoremovable Groups During Solid-Phase Synthesis of Peptides

2. Use of Photoremovable Groups During Solid-Phase Synthesis of Oligonucleotides

E. Amino Acid N-Carboxy Anhydrides Protected with a Photoremovable Group

IV. Data Collection

A. Data Collection System

B. Data Analysis

V. Other Representative Applications

A. Oligonucleotide Synthesis

1. Example

VI. Conclusion

I. DEFINITIONS

Certain terms used herein are intended to have the following general definitions:

1. Complementary:

Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

2. Epitope:

The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.

3. Ligand:

A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones, hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs (e.g. opiates, steroids, etc.), lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

4. Monomer:

A member of the set of small molecules which can be joined together to form a polymer. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of the 20 naturally occurring L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer. Furthermore, each of the sets may include protected members which are modified after synthesis.

5. Peptide:

A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer.

Peptides are often two or more amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, *Biochemistry*, Third Ed., 1988, which is incorporated herein by reference for all purposes.

6. Radiation:

Energy which may be selectively applied including energy having a wavelength of between 10^{-14} and 10^6 meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultraviolet radiation, visible light, infrared radiation, microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.

7. Receptor:

A molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex. Other examples of receptors which can be investigated by this invention include but are not restricted to:

a) Microorganism receptors:

Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in developing a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.

b) Enzymes:

For instance, one type of receptor is the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters; determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.

c) Antibodies:

For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases (e.g., by blocking the binding of the "self" antibodies).

d) Nucleic Acids:

Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.

e) Catalytic Polypeptides:

Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. Pat. No. 5,215,899, which is incorporated herein by reference for all purposes.

f) Hormone receptors:

Examples of hormones receptors include, e.g., the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which bind to a receptor may lead to the development of drugs to control blood pressure.

g) Opiate receptors:

Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

8. Substrate:

A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.

9. Protective Group:

A material which is chemically bound to a monomer unit and which may be removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protective groups with utility herein include those comprising nitropiperonyl, pyrenylmethoxy-carbonyl, nitroveratryl, nitrobenzyl, dimethyl dimethoxybenzyl, 5-bromo-7-nitroindolyl, o-hydroxy- α -methyl cinnamoyl, and 2-oxymethylene anthraquinone.

10. Predefined Region:

A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."

11. Substantially Pure:

A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.

12. Activator refers to an energy source adapted to render a group active and which is directed from a source to a predefined location on a substrate. A primary illustration of

an activator is light. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.

13. Binary Synthesis Strategy refers to an ordered strategy for parallel synthesis of diverse polymer sequences by sequential addition of reagents which may be represented by a reactant matrix, and a switch matrix, the product of which is a product matrix. A reactant matrix is a $1 \times n$ matrix of the building blocks to be added. The elements of the switch matrix are binary numbers. In preferred embodiments, a binary strategy is one in which at least two successive steps illuminate half of a region of interest on the substrate. In most preferred embodiments, binary synthesis refers to a synthesis strategy which also factors a previous addition step. For example, a strategy in which a switch matrix for a masking strategy halves regions that were previously illuminated, illuminating about half of the previously illuminated region and protecting the remaining half (while also protecting about half of previously protected regions and illuminating about half of previously protected regions). It will be recognized that binary rounds may be interspersed with non-binary rounds and that only a portion of a substrate may be subjected to a binary scheme, but will still be considered to be a binary masking scheme within the definition herein. A binary "masking" strategy is a binary synthesis which uses light to remove protective groups from materials for addition of other materials such as amino acids. In preferred embodiments, selected columns of the switch matrix are arranged in order of increasing binary numbers in the columns of the switch matrix.

14. Linker refers to a molecule or group of molecules attached to a substrate and spacing a synthesized polymer from the substrate for exposure/binding to a receptor.

II. General

The present invention provides synthetic strategies and devices for the creation of large scale chemical diversity. Solid-phase chemistry, photolabile protecting groups, and photolithography are brought together to achieve light-directed spatially-addressable parallel chemical synthesis in preferred embodiments.

The invention is described herein for purposes of illustration primarily with regard to the preparation of peptides and nucleotides, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α -, β -, or ω -amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. It will be recognized further, that illustrations herein are primarily with reference to C- to N-terminal synthesis, but the invention could readily be applied to N- to C-terminal synthesis without departing from the scope of the invention.

A. Deprotection and Addition

The present invention uses a masked light source or other activator to direct the simultaneous synthesis of many different chemical compounds. FIG. 1 is a flow chart illustrating the process of forming chemical compounds according to one embodiment of the invention. Synthesis occurs on a solid support 2. A pattern of illumination through a mask 4a using a light source 6 determines which regions of the support are activated for chemical coupling. In one preferred embodiment activation is accomplished by using light to remove photolabile protecting groups from selected areas of the substrate.

After deprotection, a first of a set of building blocks (indicated by "A" in FIG. 1), each bearing a photolabile protecting group (indicated by "X") is exposed to the surface of the substrate and it reacts with regions that were addressed by light in the preceding step. The substrate is then illuminated through a second mask 4b, which activates another region for reaction with a second protected building block "B". The pattern of masks used in these illuminations and the sequence of reactants define the ultimate products and their locations, resulting in diverse sequences at pre-defined locations, as shown with the sequences ACEG and BDFH in the lower portion of FIG. 1. Preferred embodiments of the invention take advantage of combinatorial masking strategies to form a large number of compounds in a small number of chemical steps.

A high degree of miniaturization is possible because the density of compounds is determined largely with regard to spatial addressability of the activator, in one case the diffraction of light. Each compound is physically accessible and its position is precisely known. Hence, the array is spatially-addressable and its interactions with other molecules can be assessed.

In a particular embodiment shown in FIG. 1, the substrate contains amino groups that are blocked with a photolabile protecting group. Amino acid sequences are made accessible for coupling to a receptor by removal of the photoprotective groups.

When a polymer sequence to be synthesized is, for example, a polypeptide, amino groups at the ends of linkers attached to a glass substrate are derivatized with nitroveratryloxycarbonyl (NVOC), a photoremovable protecting group. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing from 2-10 monomers, diamines, diacids, amino acids, or combinations thereof. Photodeprotection is effected by illumination of the substrate through, for example, a mask wherein the pattern has transparent regions with dimensions of, for example, less than 1 cm^2 , 10^{-1} cm^2 , 10^{-2} cm^2 , 10^{-3} cm^2 , 10^{-4} cm^2 , 10^{-5} cm^2 , 10^{-6} cm^2 , 10^{-7} cm^2 , 10^{-8} cm^2 , or 10^{-10} cm^2 . In a preferred embodiment, the regions are between about $10 \times 10 \text{ } \mu\text{m}$ and $500 \times 500 \text{ } \mu\text{m}$. According to some embodiments, the masks are arranged to produce a checkerboard array of polymers, although any one of a variety of geometric configurations may be utilized.

1. Example

In one example of the invention, free amino groups were fluorescently labelled by treatment of the entire substrate surface with fluorescein isothiocyanate (FITC) after photodeprotection. Glass microscope slides were cleaned, aminated by treatment with 0.1% aminopropyltriethoxysilane in 95% ethanol, and incubated at 110°C for 20 min. The aminated surface of the slide was then exposed to a 30 mM solution of the N-hydroxysuccinimide ester of NVOC-GABA (nitroveratryloxycarbonyl-L-amino butyric acid) in DMF. The NVOC protecting group was photolytically removed by imaging the 365 nm output from a Hg arc lamp through a chrome on glass $100 \text{ } \mu\text{m}$ checkerboard mask onto the substrate for 20 min at a power density of 12 mW/cm^2 . The exposed surface was then treated with 1 mM FITC in DMF. The substrate surface was scanned in an epifluorescence microscope (Zeiss Axioskop 20) using 488 nm excitation from an argon ion laser (Spectra-Physics model 2025). The fluorescence emission above 520 nm was detected by a cooled photomultiplier (Hamamatsu 943-02) operated in a photon counting mode. Fluorescence intensity was translated into a color display with red in the highest intensity and black in the lowest intensity areas. The pres-

ence of a high-contrast fluorescent checkerboard pattern of $100 \times 100 \mu\text{m}$ elements revealed that free amino groups were generated in specific regions by spatially localized photodeprotection.

2. EXAMPLE

FIG. 2 is a flow chart illustrating another example of the invention. Carboxy-activated NVOC-leucine was allowed to react with an aminated substrate. The carboxy activated HOBT ester of leucine and other amino acids used in this synthesis was formed by mixing 0.25 mmol of the NVOC amino protected amino acid with 37 mg HOBT (1-hydroxybenzotriazole), 111 mg BOP (benzotriazolyl-n-oxy-tris (dimethylamino)-phosphoniumhexafluorophosphate) and 86 μl DIEA (diisopropylethylamine) in 2.5 ml DMF. The NVOC protecting group was removed by uniform illumination. Carboxy-activated NVOC-phenylalanine was coupled to the exposed amino groups for 2 hours at room temperature, and then washed with DMF and methylene chloride. Two unmasked cycles of photodeprotection and coupling with carboxy-activated NVOC-glycine were carried out. The surface was then illuminated through a chrome on glass 50 μl checkerboard pattern mask. Carboxy-activated N α -tBOC-O-tButyl-L-tyrosine was then added. The entire surface was uniformly illuminated to photolyze the remaining NVOC groups. Finally, carboxy-activated NVOC-L-proline was added, the NVOC group was removed by illumination, and the t-BOC and t-butyl protecting groups were removed with TFA. After removal of the protecting groups, the surface consisted of a 50 μm checkerboard array of Tyr-Gly-Gly-Phe-Leu (YGGFL) (Seq. ID No:1) and Pro-Gly-Gly-Phe-Leu (PGGFL) (Seq. ID No:2).

B. Antibody Recognition

In one preferred embodiment the substrate is used to determine which of a plurality of amino acid sequences is recognized by an antibody of interest.

1. EXAMPLE

In one example, the array of pentapeptides in the example illustrated in FIG. 2 was probed with a mouse monoclonal antibody directed against β -endorphin. This antibody (called 3E7) is known to bind YGGFL and YGGFM (Seq. ID No:21) with nanomolar affinity and is discussed in Meo et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:4084, which is incorporated by reference herein for all purposes. This antibody requires the amino terminal tyrosine for high affinity binding. The array of peptides formed as described in FIG. 2 was incubated with a 2 $\mu\text{g/ml}$ mouse monoclonal antibody (3E7) known to recognize YGGFL. 3E7 does not bind PGGFL. A second incubation with fluoresceinated goat anti-mouse antibody labeled the regions that bound 3E7. The surface was scanned with an epi-fluorescence microscope. The results showed alternating bright and dark 50 μm squares indicating that YGGFL and PGGFL were synthesized in geometric array determined by the mask. A high contrast (>12:1 intensity ratio) fluorescence checkerboard image shows that (a) YGGFL and PGGFL were synthesized in alternate 50 μm squares, (b) YGGFL attached to the surface is accessible for binding to antibody 3E7, and (c) antibody 3E7 does not bind to PGGFL.

A three-dimensional representation of the fluorescence intensity data in a portion of the checkerboard is shown in FIG. 3. This figure shows that the border between synthesis sites is sharp. The height of each spike in this display is linearly proportional to the integrated fluorescence intensity in a 2.5 μm pixel. The transition between PGGFL and YGGFL occurs within two spikes (5 μm). There is little variation in the fluorescence intensity of different YGGFL squares. The

mean intensity of sixteen YGGFL synthesis sites was 2.03×10^5 counts and the standard deviation was 9.6×10^3 counts.

III. Synthesis

A. Reactor System

FIG. 4 schematically illustrates a device used to synthesize diverse polymer sequences on a substrate. The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment, the predefined regions on the substrate will have a surface area of between about 1 cm^2 and 10^{-10} cm^2 . In some embodiments the regions have areas of less than about 10^{-1} cm^2 , 10^{-2} cm^2 , 10^{-3} cm^2 , 10^{-4} cm^2 , 10^{-5} cm^2 , 10^{-6} cm^2 , 10^{-7} cm^2 , 10^{-8} cm^2 , 10^{-9} cm^2 or 10^{-10} cm^2 . In a preferred embodiment, the regions are between about $10 \times 10 \mu\text{m}$.

In some embodiments a single substrate supports more than about 10 different monomer sequences and preferably more than about 100 different monomer sequences, although in some embodiments more than about 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , or 10^8 different sequences are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% pure. The device includes an automated peptide synthesizer 401. The automated peptide synthesizer is a device which flows selected reagents through a flow cell 402 under the direction of a computer 404. In a preferred embodiment the synthesizer is an ABI Peptide Synthesizer, model no. 431A. The computer may be selected from a wide variety of computers or discrete logic including for example, an IBM PC-AT or similar computer linked with appropriate internal control systems in the peptide synthesizer. The PC is provided with signals from the board computer indicative of, for example, the end of a coupling cycle.

Substrate 406 is mounted on the flow cell, forming a cavity between the substrate and the flow cell. Selected reagents flow through this cavity from the peptide synthesizer at selected times, forming an array of peptides on the face of the substrate in the cavity. Mounted above the substrate, and preferably in contact with the substrate is a mask 408. Mask 408 is transparent in selected regions to a selected wavelength of light and is opaque in other regions to the selected wavelength of light. The mask is illuminated with a light source 410 such as a UV light source. In one specific embodiment the light source 410 is a model no. 82420 made by Oriel. The mask is held and translated by an x-y-z translation stage 412 such as an x-y translation stage made by Newport Corp. The computer coordinates action of the peptide synthesizer, x-y translation stage, and light source. Of course, the invention may be used in some embodiments with translation of the substrate instead of the mask.

In operation, the substrate is mounted on the reactor cavity. The slide, with its surface protected by a suitable photo removable protective group, is exposed to light at selected locations by positioning the mask and illuminating the light source for a desired period of time (such as, for example, 1 sec to 60 min in the case of peptide synthesis). A selected peptide or other monomer/polymer is pumped

through the reactor cavity by the peptide synthesizer for binding at the selected locations on the substrate. After a selected reaction time (such as about 1 sec to 300 min in the case of peptide reactions) of the monomer is washed from the system, the mask is appropriately repositioned or replaced, and the cycle is repeated. In most embodiments of the invention, reactions may be conducted at or near ambient temperature.

FIGS. 5a and 5b are flow charts of the software used in operation of the reactor system. At step 502 the peptide synthesis software is initialized. At step 504 the system calibrates positioners on the x-y translation stage and begins a main loop. At step 506 the system determines which, if any, of the function keys on the computer have been pressed. If F1 has been pressed, the system prompts the user for input of a desired synthesis process. If the user enters F2, the system allows a user to edit a file for a synthesis process at step 510. If the user enters F3 the system loads a process from a disk at step 512. If the user enters F4 the system saves an entered or edited process to disk at step 514. If the user selects F5 the current process is displayed at step 516 while selection of F6 starts the main portion of the program, i.e., the actual synthesis according to the selected process. If the user selects F7 the system displays the location of the synthesized peptides, while pressing F10 returns the user to the disk operating system.

FIG. 5b illustrates the synthesis step 518 in greater detail. The main loop of the program is started in which the system first moves the mask to a next position at step 526. During the main loop of the program, necessary chemicals flow through the reaction cell under the direction of the on-board computer in the peptide synthesizer. At step 528 the system then waits for an exposure command and, upon receipt of the exposure command, exposes the substrate for a desired time at step 530. When an acknowledge of exposure complete is received at step 532 the system determines if the process is complete at step 534 and, if so, waits for additional keyboard input at step 536 and, thereafter, exits the perform synthesis process.

A computer program used for operation of the system described above is included as microfiche Appendix A (Copyright, 1990, Affymax Technologies N.V., all rights reserved). The program is written in Turbo C++ (Borland Int'l) and has been implemented in an IBM compatible system. The motor control software is adapted from software produced by Newport Corporation. It will be recognized that a large variety of programming languages could be utilized without departing from the scope of the invention herein. Certain calls are made to a graphics program in "Programmer Guide to PC and PS2 Video Systems" (Wilton, Microsoft Press, 1987), which is incorporated herein by reference for all purposes.

Alignment of the mask is achieved by one of two methods in preferred embodiments. In a first embodiment the system relies upon relative alignment of the various components, which is normally acceptable since x-y-z translation stages are capable of sufficient accuracy for the purposes herein. In alternative embodiments, alignment marks on the substrate are coupled to a CCD device for appropriate alignment.

According to some embodiments, pure reagents are not added at each step, or complete photolysis of the protective groups is not provided at each step. According to these embodiments, multiple products will be formed in each synthesis site. For example, if the monomers A and B are mixed during a synthesis step, A and B will bind to deprotected regions, roughly in proportion to their concentration in solution. Hence, a mixture of compounds will be formed

in a synthesis region. A substrate formed with mixtures of compounds in various synthesis regions may be used to perform, for example, an initial screening of a large number of compounds, after which a smaller number of compounds in regions which exhibit high binding affinity are further screened. Similar results may be obtained by only partially photolyzing a region, adding a first monomer, re-photolyzing the same region, and exposing the region to a second monomer.

B. Binary Synthesis Strategy

In a light-directed chemical synthesis, the products formed depend on the pattern and order of masks, and on the order of reactants. To make a set of products there will in general be "n" possible masking schemes. In preferred embodiments of the invention herein a binary synthesis strategy is utilized. The binary synthesis strategy is illustrated herein primarily with regard to a masking strategy, although it will be applicable to other polymer synthesis strategies such as the pin strategy, and the like.

In a binary synthesis strategy, the substrate is irradiated with a first mask, exposed to a first building block, irradiated with a second mask, exposed to a second building block, etc. Each combination of masked irradiation and exposure to a building block is referred to herein as a "cycle."

In a preferred binary masking scheme, the masks for each cycle allow irradiation of half of a region of interest on the substrate and protection of the remaining half of the region of interest. By "half" it is intended herein not to mean exactly one-half the region of interest, but instead a large fraction of the region of interest such as from about 30 to 70 percent of the region of interest. It will be understood that the entire masking scheme need not take a binary form; instead non-binary cycles may be introduced as desired between binary cycles.

In preferred embodiments of the binary masking scheme, a given cycle illuminates only about half of the region which was illuminated in a previous cycle, while protecting the remaining half of the illuminated portion from the previous cycle. Conversely, in such preferred embodiments, a given cycle illuminates half of the region which was protected in the previous cycle and protects half the region which was protected in a previous cycle.

The synthesis strategy is most readily illustrated and handled in matrix notation. At each synthesis site, the determination of whether to add a given monomer is a binary process. Therefore, each product element P_j is given by the dot product of two vectors, a chemical reactant vector, e.g., $C=[A,B,C,D]$, and a binary vector σ_j . Inspection of the products in the example below for a four-step synthesis, shows that in one four-step synthesis $\sigma_1=[1,0,1,0]$, $\sigma_2=[1,0,0,1]$, $\sigma_3=[0,1,1,0]$, and $\sigma_4=[0,1,0,1]$, where a 1 indicates illumination and a 0 indicates protection. Therefore, it becomes possible to build a "switch matrix" S from the column vectors σ_j ($j=1,k$ where k is the number of products).

$$\begin{array}{cccc}
 \sigma_1 & \sigma_2 & \sigma_3 & \sigma_4 \\
 1 & 1 & 0 & 0 \\
 S=0 & 0 & 1 & 1 \\
 1 & 0 & 1 & 0 \\
 0 & 1 & 0 & 1
 \end{array}$$

The outcome P of a synthesis is simply $P=CS$, the product of the chemical reactant matrix and the switch matrix.

The switch matrix for an n-cycle synthesis yielding k products has n rows and k columns. An important attribute of S is that each row specifies a mask. A two-dimensional mask m_j for the jth chemical step of a synthesis is obtained

directly from the j th row of S by placing the elements s_{jk} , \dots , s_{jk} into, for example, a square format. The particular arrangement below provides a square format, although linear or other arrangements may be utilized.

$$S = \begin{matrix} & s_{11} & s_{12} & s_{13} & s_{14} & & \\ s_{21} & s_{22} & s_{23} & s_{24} & s_{25} & s_{26} & \\ s_{31} & s_{32} & s_{33} & s_{34} & s_{35} & s_{36} & \\ & s_{41} & s_{42} & s_{43} & s_{44} & & \end{matrix}$$

Of course, compounds formed in a light-activated synthesis can be positioned in any defined geometric array. A square or rectangular matrix is convenient but not required. The rows of the switch matrix may be transformed into any convenient array as long as equivalent transformations are used for each row.

For example, the masks in the four-step synthesis below are then denoted by:

$$m_1 = \begin{matrix} 1 & 1 & 0 & 0 & 1 & 0 & 0 & 1 \\ 0 & 0 & 1 & 1 & 1 & 0 & 0 & 1 \end{matrix}$$

where 1 denotes illumination (activation) and 0 denotes no illumination.

The matrix representation is used to generate a desired set of products and product maps in preferred embodiments. Each compound is defined by the product of the chemical vector and a particular switch vector. Therefore, for each synthesis address, one simply saves the switch vector, assembles all of them into a switch matrix, and extracts each of the rows to form the masks.

In some cases, particular product distributions or a maximum number of products are desired. For example, for $C=[A,B,C,D]$, any switch vector (σ) consists of four bits. Sixteen four-bit vectors exist. Hence, a maximum of 16 different products can be made by sequential addition of the reagents $[A,B,C,D]$. These 16 column vectors can be assembled in 16! different ways to form a switch matrix. The order of the column vectors defines the masking patterns, and, therefore, the spatial ordering of products but not their makeup. One ordering of these columns gives the following switch matrix (in which "null" (\emptyset) additions are included in brackets for the sake of completeness, although such null additions are elsewhere ignored herein):

$$S = \begin{matrix} \sigma_1 & & & & & & & & & & \sigma_M & & & & & & \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & A \\ [0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1] & \emptyset \\ 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & B \\ [0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1] & \emptyset \\ 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & C \\ [0 & 0 & 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & 1 & 1] & \emptyset \\ 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & D \\ [0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1] & \emptyset \end{matrix}$$

The columns of S according to this aspect of the invention are the binary representations of the numbers 15 to 0. The sixteen products of this binary synthesis are ABCD, ABC, ABD, AB, ACD, AC, AD, A, BCD, BC, BD, B, CD, C, D, and \emptyset (null). Also note that each of the switch vectors from the four-step synthesis masks above (and hence the synthesis products) are present in the four bit binary switch matrix. (See columns 6, 7, 10, and 11)

This synthesis procedure provides an easy way for mapping the completed products. The products in the various

locations on the substrate are simply defined by the columns of the switch matrix (the first column indicating, for example, that the product ABCD will be present in the upper left-hand location of the substrate). Furthermore, if only selected desired products are to be made, the mask sequence can be derived by extracting the columns with the desired sequences. For example, to form the product set ABCD, ABD, ACD, AD, BCD, BD, CD, and D, the masks are formed by use of a switch matrix with only the 1st, 3rd, 5th, 7th, 9th, 11th, 13th, and 15th columns arranged into the switch matrix:

$$S = \begin{matrix} 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \end{matrix}$$

To form all of the polymers of length 4, the reactant matrix [ABCDABCDABCDABCD] is used. The switch matrix will be formed from a matrix of the binary numbers from 0 to 2^{16} arranged in columns. The columns having four monomers are then selected and arranged into a switch matrix. Therefore, it is seen that the binary switch matrix in general will provide a representation of all the products which can be made from an n -step synthesis, from which the desired products are then extracted.

The rows of the binary switch matrix will, in preferred embodiments, have the property that each masking step illuminates half of the synthesis area. Each masking step also factors the preceding masking step; that is, half of the region that was illuminated in the preceding step is again illuminated, whereas the other half is not. Half of the region that was unilluminated in the preceding step is also illuminated, whereas the other half is not. Thus, masking is recursive. The masks are constructed, as described previously, by extracting the elements of each row and placing them in a square array. For example, the four masks in S for a four-step synthesis are:

$$m_1 = \begin{matrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{matrix}$$

$$m_2 = \begin{matrix} 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \end{matrix}$$

The recursive factoring of masks allows the products of a light-directed synthesis to be represented by a polynomial. (Some light activated syntheses can only be denoted by irreducible, i.e., prime polynomials.) For example, the polynomial corresponding to the top synthesis of FIG. 9a (discussed below) is

$$P=(A+B)C+D$$

A reaction polynomial may be expanded as though it were an algebraic expression, provided that the order of joining of reactants X_1 and X_2 is preserved ($X_1X_2 \neq X_2X_1$), i.e., the products are not commutative. The product then is $AC+AD+BC+BD$. The polynomial explicitly specifies the reactants and implicitly specifies the mask for each step. Each pair of parentheses demarcates a round of synthesis. The chemical reactants of a round (e.g., A and B) react at nonoverlapping sites and hence cannot combine with one other. The synthe-

sis area is divided equally amongst the elements of a round (e.g., A is directed to one-half of the area and B to the other half). Hence, the masks for a round (e.g., the masks m_A and m_B) are orthogonal and form an orthonormal set. The polynomial notation also signifies that each element in a round is to be joined to each element of the next round (e.g., A with C, A with D, B with C, and B with D). This is accomplished by having m_C overlap m_A and m_D equally, and likewise for m_D . Because C and D are elements of a round, m_C and m_D are orthogonal to each other and form an orthonormal set.

The polynomial representation of the binary synthesis described above, in which 16 products are made from 4 reactants, is

$$P=(A+\theta)(B+\theta)(C+\theta)(D+\theta)$$

which gives ABCD, ABC, ABD, AB, ACD, AC, AD, A, BCD, BC, BD, B, CD, C, D, and θ when expanded (with the rule that $\theta X=X$ and $X\theta=X$, and remembering that joining is ordered). In a binary synthesis, each round contains one reactant and one null (denoted by θ). Half of the synthesis area receives the reactant and the other half receives nothing. Each mask overlaps every other mask equally.

Binary rounds and non-binary rounds can be interspersed as desired, as in

$$P=(A+\theta)(B)(C+D+\theta)(E+F+\theta)$$

The 18 compounds formed are ABCE, ABCF, ABCG, ABDE, ABDF, ABDF, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE, ABDE. The switch matrix S for this 7-step synthesis is

1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
S=0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0
1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0
0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0
0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1

The round denoted by (B) places B in all products because the reaction area was uniformly activated (the mask for B consisted entirely of 1's).

The number of compounds k formed in a synthesis consisting of r rounds, in which the i th round has b_i chemical reactants and z_i nulls, is

$$k=2^{(b+z)}$$

and the number of chemical steps n is

$$n=\sum b_i$$

The number of compounds synthesized when $b=a$ and $z=0$ in all rounds is a^{2^r} , compared with 2^n for a binary synthesis. For $n=20$ and $a=5$, 625 compounds (all tetramers) would be formed, compared with 1.049×10^6 compounds in a binary synthesis with the same number of chemical steps.

It should also be noted that rounds in a polynomial can be nested, as in

$$(A+(B+\theta)(C+\theta)(D+\theta))$$

The products are AD, BCD, BD, CD, D, A, BC, B, C, and θ .

Binary syntheses are attractive for two reasons. First, they generate the maximal number of products (2^n) for a given

number of chemical steps (n). For four reactants, 16 compounds are formed in the binary synthesis, whereas only 4 are made when each round has two reactants. A 10-step binary synthesis yields 1,024 compounds, and a 20-step synthesis yields 1,048,576. Second, products formed in a binary synthesis are a complete nested set with lengths ranging from 0 to n . All compounds that can be formed by deleting one or more units from the longest product (the n -mer) are present. Contained within the binary set are the smaller sets that would be formed from the same reactants using any other set of masks (e.g., AC, AD, BC, and BD formed in the synthesis shown in FIG. 6 are present in the set of 16 formed by the binary synthesis). In some cases, however, the experimentally achievable spatial resolution may not suffice to accommodate all the compounds formed. Therefore, practical limitations may require one to select a particular subset of the possible switch vectors for a given synthesis.

1. EXAMPLE

FIG. 6 illustrates a synthesis with binary masking scheme.

The binary masking scheme provides the greatest number of sequences for a given number of cycles. According to this embodiment, a mask m_1 allows illumination of half of the substrate. The substrate is then exposed to the building block A, which binds at the illuminated regions.

Thereafter, the mask m_2 allows illumination of half of the previously illuminated region, while protecting half of the previously illuminated region. The building block B is then added, which binds at the illuminated regions from m_2 .

The process continues with masks m_3 , m_4 , and m_5 , resulting in the product array shown in the bottom portion of the figure. The process generates 32 (2^5) sequences with 5 (the number of monomers) cycles.

2. EXAMPLE

FIG. 7 illustrates another preferred binary masking scheme which is referred to herein as the gray code masking scheme. According to this embodiment, the masks m_1 to m_5 are selected such that a side of any given synthesis region is defined by the edge of only one mask. The site at which the sequence BCDE is formed, for example, has its right edge defined by m_5 and its left side formed by mask m_4 (and no other mask is aligned on the sides of this site). Accordingly, problems created by misalignment, diffusion of light under the mask and the like will be minimized.

3. EXAMPLE

FIG. 8 illustrates another binary masking scheme. According to this scheme, referred to herein as a modified gray code masking scheme, the number of masks needed is minimized. For example, the mask m_2 could be the same mask as m_1 and simply translated laterally. Similarly, the mask m_4 could be the same as mask m_3 and simply translated laterally.

4. EXAMPLE

A four-step synthesis is shown in FIG. 9a. The reactants are the ordered set {A,B,C,D}. In the first cycle, illumination through m_1 activates the upper half of the synthesis area. Building block A is then added to give the distribution 602. Illumination through mask m_2 (which activates the lower half), followed by addition of B yields the next intermediate distribution 604. C is added after illumination through m_3 (which activates the left half) giving the distribution 604, and D after illumination through m_4 (which activates the right half), to yield the final product pattern 608 (ACAD, BCBD).

5. EXAMPLE

The above masking strategy for the synthesis may be extended for all 400 dipeptides from the 20 naturally occur-

ring amino acids as shown in FIG. 9b. The synthesis consists of two rounds, with 20 photolysis and chemical coupling cycles per round. In the first cycle of round 1, mask 1 activates 1/20th of the substrate for coupling with the first of 20 amino acids. Nineteen subsequent illumination/coupling cycles in round 1 yield a substrate consisting of 20 rectangular stripes each bearing a distinct member of the 20 amino acids. The masks of round 2 are perpendicular to round 1 masks and therefore a single illumination/coupling cycle in round 2 yields 20 dipeptides. The 20 illumination/coupling cycles of round 2 complete the synthesis of the 400 dipeptides.

6. EXAMPLE

The power of the binary masking strategy can be appreciated by the outcome of a 10-step synthesis that produced 1,024 peptides. The polynomial expression for this 10-step binary synthesis was:

$$(1+0)(Y+0)(G+0)(A+0)(C+0)(T+0)(F+0)(L+0)(S+0)(F+0)$$

Each peptide occupied a 400x400 μm square. A 32x32 peptide array (1,024 peptides, including the null peptide and 10 peptides of $l=1$, and a limited number of duplicates) was clearly evident in a fluorescence scan following side group deprotection and treatment with the antibody 3E7 and fluoresceinated antibody. Each synthesis site was a 400x400 μm square.

The scan showed a range of fluorescence intensities, from a background value of 3,300 counts to 22,400 counts in the brightest square ($x=20, y=9$). Only 15 compounds exhibited an intensity greater than 12,300 counts. The median value of the array was 4,800 counts.

The identity of each peptide in the array could be determined from its x and y coordinates (each range from 0 to 31) and the map of FIG. 10. The chemical units at positions 2, 5, 6, 9, and 10 are specified by the y coordinate and those at positions 1, 3, 4, 7, 8 by the x coordinate. All but one of the peptides was shorter than 10 residues. For example, the peptide at $x=12$ and $y=3$ is YGAGF (SEQ. ID No:3) (positions 1, 6, 8, 9, and 10 are nulls). YGAFLS (SEQ. ID No:4), the brightest element of the array, is at $x=20$ and $y=9$.

It is often desirable to deduce a binding affinity of a given peptide from the measured fluorescence intensity. Conceptually, the simplest case is one in which a single peptide binds to a univalent antibody molecule. The fluorescence scan is carried out after the slide is washed with buffer for a defined time. The order of fluorescence intensities is then a measure primarily of the relative dissociation rates of the antibody-peptide complexes. If the on-rate constants are the same (e.g., if they are diffusion-controlled), the order of fluorescence intensities will correspond to the order of binding affinities. However, the situation is sometimes more complex because a bivalent primary antibody and a bivalent secondary antibody are used. The density of peptides in a synthesis area corresponded to a mean separation of ~ 7 nm, which would allow multivalent antibody-peptide interactions. Hence, fluorescence intensities obtained according to the method herein will often be a qualitative indicator of binding affinity.

Another important consideration is the fidelity of synthesis. Deletions are produced by incomplete photodeprotection or incomplete coupling. The coupling yield per cycle in these experiments is typically between 85% and 95%. Implementing the switch matrix by masking is imperfect because of light diffraction, internal reflection, and scattering. Consequently, stowaways (chemical units that should not be on board) arise by unintended illumination of regions that should be dark. A binary synthesis array contains many

of the controls needed to assess the fidelity of a synthesis. For example, the fluorescence signal from a synthesis area nominally containing a tetrapeptide ABCD could come from a tripeptide deletion impurity such as ACD. Such an artifact would be ruled out by the finding that the fluorescence intensity of the ACD-site is less than that of the ABCD site.

The fifteen most highly labelled peptides in the array obtained with the synthesis of 1,024 peptides described above, were YGAFLS (SEQ. ID No:5), YGAFLS (SEQ. ID No:6), YGAFL (SEQ. ID No:7), YGGFLS (SEQ. ID No:8), YGAF (SEQ. ID No:8), YGALS (SEQ. ID No:9), YGGFS (SEQ. ID No:10), YGAL (SEQ. ID No:11), YGAFLF (SEQ. ID No:12), YGAF (SEQ. ID No:13), YGAFF (SEQ. ID No:14), YGGLS (SEQ. ID No:15), YGGFL (SEQ. ID No:16), SEQ. ID No:17, and YGAFLSF (SEQ. ID No:17). I fifteen begin with YG, which agrees with previous work showing that an amino-terminal tyrosine is a key determinant of binding. Residue 3 of this set is either A or G, and residue 4 is either F or L. The exclusion of S and T from these positions is clear cut. The finding that the preferred sequence is YG (A/G) (F/L) fits nicely with the outcome of a study in which a very large library of peptides on phage generated by recombinant DNA methods was screened for binding to antibody 3E7 (see Cwirla et al., *Proc. Natl. Acad. Sci. USA.* (1990) 87:6378, incorporated herein by reference). Additional binary syntheses based on leads from peptides on phage experiments show that YGAFLMQ (SEQ. ID No:18), YGAFLM (SEQ. ID No:19), and YGAFLQ (SEQ. ID No:20) give stronger fluorescence signals than does YGGFLM, the immunogen used to obtain antibody 3E7.

Variations on the above masking strategy will be valuable in certain circumstances. For example, if a "kernel" sequence of interest consists of PQR separated from XYZ and that the aim is to synthesize peptides in which these units are separated by a variable number of different residues, then the kernel can be placed in each peptide by using a mask that has 1's everywhere. The polynomial representation of a suitable synthesis is:

$$(P)(X)(R)(X)(A+0)(B+0)(C+0)(D+0)(X)(Y)(Z)$$

Sixteen peptides will be formed, ranging in length from the 6-mer PQRXYZ to the 10-mer PQRABCDXYZ.

Several other masking strategies will also find value in selected circumstances. By using a particular mask more than once, two or more reactants will appear in the same set of products. For example, suppose that the mask for an 8-step synthesis is

A	11110000
B	00001111
C	11001100
D	00110011
E	10101010
F	01010101
G	11110000
H	00001111

The products are ACEG, ACFG, ADEG, ADFG, BCEH, BCFH, BDEH, and BDFH. A and G always appear together because their additions were directed by the same mask, and likewise for B and H.

C. Linker Selection

According to preferred embodiments the linker molecules used as an intermediary between the synthesized polymers and the substrate are selected for optimum length and/or type for improved binding interaction with a receptor. According to this aspect of the invention diverse linkers of

varying length and/or type are synthesized for subsequent attachment of a ligand. Through variations in the length and type of linker, it becomes possible to optimize the binding interaction between an immobilized ligand and its receptor.

The degree of binding between a ligand (peptide, inhibitor, hapten, drug, etc.) and its receptor (enzyme, antibody, etc.) when one of the partners is immobilized on to a substrate will in some embodiments depend on the accessibility of the receptor in solution to the immobilized ligand. The accessibility in turn will depend on the length and/or type of linker molecule employed to immobilize one of the partners. Preferred embodiments of the invention therefore employ the ULSIPS™ technology described herein to generate an array of, preferably, inactive or inert linkers of varying length and/or type, using photochemical protecting groups to selectively expose different regions of the substrate and to build upon chemically-active groups.

In the simplest embodiment of this concept, the same unit is attached to the substrate in varying multiples or lengths in known locations on the substrate via VLSIPS™ techniques to generate an array of polymers of varying length. A single ligand (peptide, drug, hapten, etc.) is attached to each of them, and an assay is performed with the binding site to evaluate the degree of binding with a receptor that is known to bind to the ligand. In cases where the linker length impacts the ability of the receptor to bind to the ligand, varying levels of binding will be observed. In general, the linker which provides the highest binding will then be used to assay other ligands synthesized in accordance with the techniques herein.

According to other embodiments the binding between a single ligand/receptor pair is evaluated for linkers of diverse monomer sequence. According to these embodiments, the linkers are synthesized in an array in accordance with the techniques herein and have different monomer sequence (and, optionally, different lengths). Thereafter, all of the linker molecules are provided with a ligand known to have at least some binding affinity for a given receptor. The given receptor is then exposed to the ligand and binding affinity is deduced. Linker molecules which provide adequate binding between the ligand and receptor are then utilized in screening studies.

D. Protecting Groups

As discussed above, selectively removable protecting groups allow creation of well defined areas of substrate surface having differing reactivities. Preferably, the protecting groups are selectively removed from the surface by applying a specific activator, such as electromagnetic radiation of a specific wavelength and intensity. More preferably, the specific activator exposes selected areas of surface to remove the protecting groups in the exposed areas.

Protecting groups of the present invention are used in conjunction with solid phase oligomer syntheses, such as peptide syntheses using natural or unnatural amino acids, nucleotide syntheses using deoxyribonucleic and ribonucleic acids, oligosaccharide syntheses, and the like. In addition to protecting the substrate surface from unwanted reaction, the protecting groups block a reactive end of the monomer to prevent self-polymerization. For instance, attachment of a protecting group to the amino terminus of an activated amino acid, such as an N-hydroxysuccinimide-activated ester of the amino acid, prevents the amino terminus of one monomer from reacting with the activated ester portion of another during peptide synthesis. Alternatively, the protecting group may be attached to the carboxyl group of an amino acid to prevent reaction at this site. Most protecting groups can be attached to either the amino or the

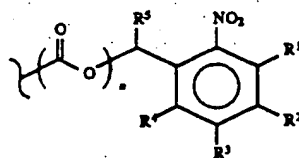
carboxyl group of an amino acid, and the nature of the chemical synthesis will dictate which reactive group will require a protecting group. Analogously, attachment of a protecting group to the 5'-hydroxyl group of a nucleoside during synthesis using for example, phosphate-triester coupling chemistry, prevents the 5'-hydroxyl of one nucleoside from reacting with the 3'-activated phosphate-triester of another.

Regardless of the specific use, protecting groups are employed to protect a moiety on a molecule from reacting with another reagent. Protecting groups of the present invention have the following characteristics: they prevent selected reagents from modifying the group to which they are attached; they are stable (that is, they remain attached to the molecule) to the synthesis reaction conditions; they are removable under conditions that do not adversely affect the remaining structure; and once removed, do not react appreciably with the surface or surface-bound oligomer. The selection of a suitable protecting group will depend, of course, on the chemical nature of the monomer unit and oligomer, as well as the specific reagents they are to protect against.

In a preferred embodiment, the protecting groups are photoactivatable. The properties and uses of photoreactive protecting compounds have been reviewed. See, McCray et al., *Ann. Rev. of Biophys. and Biophys. Chem.* (1989) 18:239-270, which is incorporated herein by reference. Preferably, the photosensitive protecting groups will be removable by radiation in the ultraviolet (UV) or visible portion of the electromagnetic spectrum. More preferably, the protecting groups will be removable by radiation in the near UV or visible portion of the spectrum. In some embodiments, however, activation may be performed by other methods such as localized heating, electron beam lithography, laser pumping, oxidation or reduction with microelectrodes, and the like. Sulfonyl compounds are suitable reactive groups for electron beam lithography. Oxidative or reductive removal is accomplished by exposure of the protecting group to an electric current source, preferably using microelectrodes directed to the predefined regions of the surface which are desired for activation. Other methods may be used in light of this disclosure.

Many, although not all, of the photoremovable protecting groups will be aromatic compounds that absorb near-UV and visible radiation. Suitable photoremovable protecting groups are described in, for example, McCray et al., *Patchornik, J. Amer. Chem. Soc.* (1970) 92 :6333, and Amit et al., *J. Org. Chem.* (1974) 39:192, which are incorporated herein by reference.

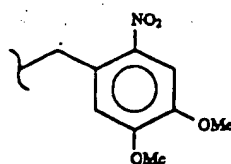
A preferred class of photoremovable protecting groups has the general formula:



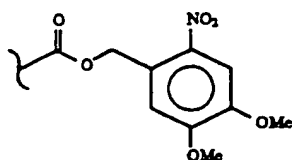
where R^1 , R^2 , R^3 , and R^4 independently are a hydrogen atom, a lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxy, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido group, or adjacent substituents (i.e., R^1-R^2 , R^2-R^3 , R^3-R^4) are substituted oxygen groups that together form an cyclic acetal or ketal; R^5 is a hydrogen atom, a alkoxy, alkyl, hydroxyl, halo, aryl, or alkenyl group, and $n=0$ or 1.

21

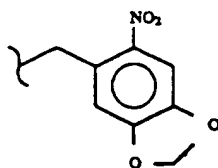
A preferred protecting group, 6-nitroveratryl (NV), which is used for protecting the carboxyl terminus of an amino acid or the hydroxyl group of a nucleotide, for example, is formed when R^2 and R^3 are each a methoxy group, R^1 , R^4 and R^5 are each a hydrogen atom, and $n=0$:



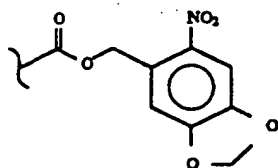
A preferred protecting group, 6-nitroveratryloxycarbonyl (NVOC), which is used to protect the amino terminus of an amino acid, for example, is formed when R^2 and R^3 are each a methoxy group, R^1 , R^4 and R^5 are each a hydrogen atom, and $n=1$:



Another preferred protecting group, 6-nitropiperonyl (NP), which is used for protecting the carboxyl terminus of an amino acid or the hydroxyl group of a nucleotide, for example, is formed when R^2 and R^3 together form a methylene acetal, R^1 , R^4 and R^5 are each a hydrogen atom, and $n=0$:

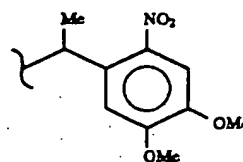


Another preferred protecting group, 6-nitropiperonyloxycarbonyl (NPOC), which is used to protect the amino terminus of an amino acid, for example, is formed when R^2 and R^3 together form a methylene acetal, R^1 , R^4 and R^5 are each a hydrogen atom, and $n=1$:

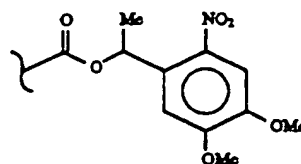


A most preferred protecting group, methyl-6-nitroveratryl (MeNV), which is used for protecting the carboxyl terminus of an amino acid or the hydroxyl group of a nucleotide, for example, is formed when R^2 and R^3 are each a methoxy group, R^1 and R^4 are each a hydrogen atom, R^5 is a methyl group, and $n=0$:

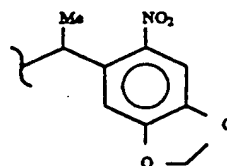
22



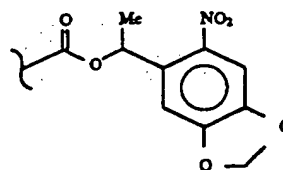
Another most preferred protecting group, methyl-6-nitroveratryloxycarbonyl (MeNVOC), which is used to protect the amino terminus of an amino acid, for example, is formed when R^2 and R^3 are each a methoxy group, R^1 and R^4 are each a hydrogen atom, R^5 is a methyl group, and $n=1$:



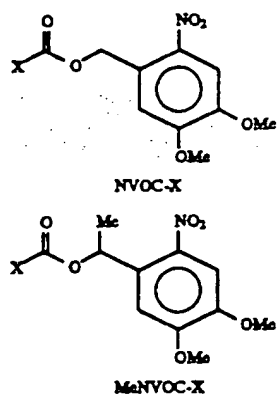
Another most preferred protecting group, methyl-6-nitropiperonyl (MeNP), which is used for protecting the carboxyl terminus of an amino acid or the hydroxyl group of a nucleotide, for example, is formed when R^2 and R^3 together form a methylene acetal, R^1 and R^4 are each a hydrogen atom, R^5 is a methyl group, and $n=0$:



Another most preferred protecting group, methyl-6-nitropiperonyloxycarbonyl (MeNPOC), which is used to protect the amino terminus of an amino acid, for example, is formed when R^2 and R^3 together form a methylene acetal, R^1 and R^4 are each a hydrogen atom, R^5 is a methyl group, and $n=1$:

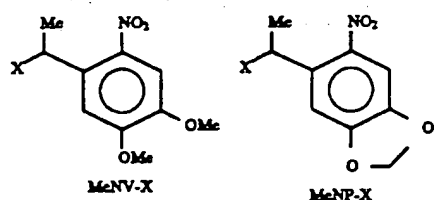


A protected amino acid having a photoactivatable oxycarbonyl protecting group, such as NVOC or NPOC or their corresponding methyl derivatives, MeNVOC or MeNPOC, respectively, on the amino terminus is formed by acylating the amine of the amino acid with an activated oxycarbonyl ester of the protecting group. Examples of activated oxycarbonyl esters of NVOC and MeNVOC have the general formula:



where X is halogen, mixed anhydride, phenoxy, 20
p-nitrophenoxy, N-hydroxysuccinimide, and the like.

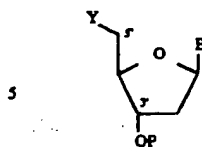
A protected amino acid or nucleotide having a photoacti-
vatable protecting group, such as NV or NP or their
corresponding methyl derivatives, MeNV or MeNP, respectively, on the carboxy terminus of the amino acid or
5'-hydroxy terminus of the nucleotide, is formed by acylat-
ing the carboxy terminus or 5'-OH with an activated benzyl
derivative of the protecting group. Examples of activated
benzyl derivatives of MeNV and MeNP have the general
formula:



where X is halogen, hydroxyl, tosyl, mesyl, trifluoromethyl,
diaz, azido, and the like.

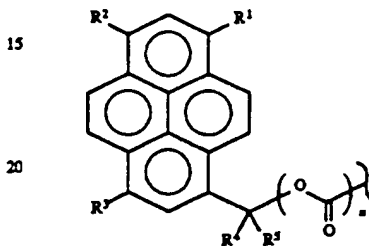
Another method for generating protected monomers is to
react the benzylic alcohol derivative of the protecting group
with an activated ester of the monomer. For example, to
protect the carboxyl terminus of an amino acid, an activated
ester of the amino acid is reacted with the alcohol derivative
of the protecting group, such as 6-nitroveratrol (NVOH).
Examples of activated esters suitable for such uses include
halo-formate, mixed anhydride, imidazolyl formate acyl
halide, and also includes formation of the activated ester in
situ the use of common reagents such as DCC and the like.
See Atherton et al. for other examples of activated esters.

A further method for generating protected monomers is to
react the benzylic alcohol derivative of the protecting group
with an activated carbon of the monomer. For example, to
protect the 5'-hydroxyl group of a nucleic acid, a derivative
having a 5'-activated carbon is reacted with the alcohol
derivative of the protecting group, such as methyl-6-
nitropiperonol (MePyROH). Examples of nucleotides hav-
ing activating groups attached to the 5'-hydroxyl group have
the general formula:



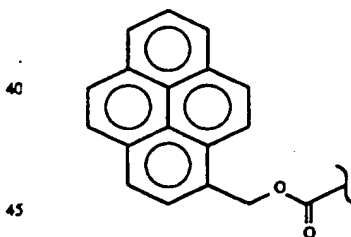
where Y is a halogen atom, a tosyl, mesyl, trifluoromethyl,
azido, or diazo group, and the like.

Another class of preferred photochemical protecting
groups has the formula:

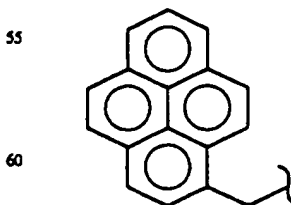


where R¹, R², and R³ independently are a hydrogen atom, a
lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxy, thiol,
thioether, amino, nitro, carboxyl, formate, formamido,
sulfonates, sulfido or phosphido group, R⁴ and R⁵ indepen-
dently are a hydrogen atom, an alkoxy, alkyl, halo, aryl,
hydrogen, or alkenyl group, and n=0 or 1.

A preferred protecting group,
1-pyrenylmethyloxycarbonyl (PyROC), which is used to
protect the amino terminus of an amino acid, for example, is
formed when R¹ through R⁵ are each a hydrogen atom and
n=1:

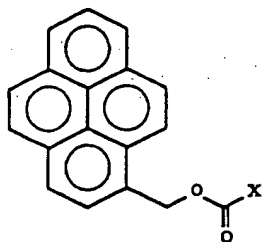


Another preferred protecting group, 1-pyrenylmethyl
(PyR), which is used for protecting the carboxy terminus of
an amino acid or the hydroxyl group of a nucleotide, for
example, is formed when R¹ through R⁵ are each a hydrogen
atom and n=0:



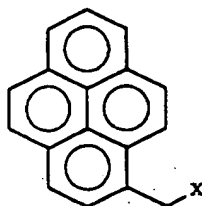
An amino acid having a pyrenylmethyloxycarbonyl pro-
tecting group on its amino terminus is formed by acylation
of the free amine of amino acid with an activated oxycar-
bonyl ester of the pyrenyl protecting group. Examples of

activated oxycarbonyl esters of PyROC have the general formula:



where X is halogen, or mixed anhydride, p-nitrophenoxo, or N-hydroxysuccinimide group, and the like.

A protected amino acid or nucleotide having a photoactivatable protecting group, such as PyR, on the carboxy terminus of the amino acid or 5'-hydroxy terminus of the nucleic acid, respectively, is formed by acylating the carboxy terminus or 5'-OH with an activated pyrenylmethyl derivative of the protecting group. Examples of activated pyrenylmethyl derivatives of PyR have the general formula:



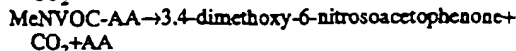
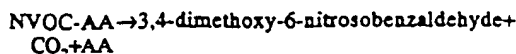
where X is a halogen atom, a hydroxyl, diazo, or azido group, and the like.

Another method of generating protected monomers is to react the pyrenylmethyl alcohol moiety of the protecting group with an activated ester of the monomer. For example, an activated ester of an amino acid can be reacted with the alcohol derivative of the protecting group, such as pyrenylmethyl alcohol (PyROH), to form the protected derivative of the carboxy terminus of the amino acid. Examples of activated esters include halo-formate, mixed anhydride, imidazolyl formate, acyl halide, and also includes formation of the activated ester in situ and the use of common reagents such as DCC and the like.

Clearly, many photosensitive protecting groups are suitable for use in the present invention.

In preferred embodiments, the substrate is irradiated to remove the photoremovable protecting groups and create regions having free reactive moieties and side products resulting from the protecting group. The removal rate of the protecting groups depends on the wavelength and intensity of the incident radiation, as well as the physical and chemical properties of the protecting group itself. Preferred protecting groups are removed at a faster rate and with a lower intensity of radiation. For example, at a given set of conditions, MeNVOC and MeNPOC are photolytically removed from the N-terminus of a peptide chain faster than their unsubstituted parent compounds, NVOC and NPOC, respectively.

Removal of the protecting group is accomplished by irradiation to liberate the reactive group and degradation products derived from the protecting group. Not wishing to be bound by theory, it is believed that irradiation of an NVOC- and MeNVOC-protected oligomers occurs by the following reaction schemes:



where AA represents the N-terminus of the amino acid oligomer.

Along with the unprotected amino acid, other products are liberated into solution: carbon dioxide and a 2,3-dimethoxy-6-nitrosophenylcarbonyl compound, which can react with nucleophilic portions of the oligomer to form unwanted secondary reactions. In the case of an NVOC-protected amino acid, the degradation product is a nitrosobenzaldehyde, while the degradation product for the other is a nitrosophenyl ketone. For instance, it is believed that the product aldehyde from NVOC degradation reacts with free amines to form a Schiff base (imine) that affects the remaining polymer synthesis. Preferred photoremovable protecting groups react slowly or reversibly with the oligomer on the support.

Again not wishing to be bound by theory, it is believed that the product ketone from irradiation of a MeNVOC-protected oligomer reacts at a slower rate with nucleophiles on the oligomer than the product aldehyde from irradiation of the same NVOC-protected oligomer. Although not unambiguously determined, it is believed that this difference in reaction rate is due to the difference in general reactivity between aldehyde and ketones towards nucleophiles due to steric and electronic effects.

The photoremovable protecting groups of the present invention are readily removed. For example, the photolysis of N-protected L-phenylalanine in solution and having different photoremovable protecting groups was analyzed, and the results are presented in the following table:

TABLE

Photolysis of Protected L-Phe-OH

Solvent	t _{1/2} in seconds			
	NBOC	NVOC	MeNVOC	MeNPOC
Dioxane	1288	110	24	19
5 mM H ₂ SO ₄ /Dioxane	1575	98	33	22

The half life, t_{1/2}, is the time in seconds required to remove 50% of the starting amount of protecting group. NBOC is the 6-nitrobenzyloxycarbonyl group, NVOC is the 6-nitroveratryloxycarbonyl group, MeNVOC is the methyl-6-nitroveratryloxycarbonyl group, and MeNPOC is the methyl-6-nitropiperonyloxycarbonyl group. The photolysis was carried out in the indicated solvent with 362/364 nm-wavelength irradiation having an intensity of 10 mW/cm², and the concentration of each protected phenylalanine was 0.10 mM.

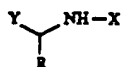
The table shows that deprotection of NVOC-, MeNVOC-, and MeNPOC-protected phenylalanine proceeded faster than the deprotection of NBOC. Furthermore, it shows that the deprotection of the two derivatives that are substituted on the benzylic carbon, MeNVOC and MeNPOC, were photolyzed at the highest rates in both dioxane and acidified dioxane.

1. Use of Photoremovable Groups During Solid-Phase Synthesis of Peptides

The formation of peptides on a solid-phase support requires the stepwise attachment of an amino acid to a substrate-bound growing chain. In order to prevent unwanted polymerization of the monomeric amino acid under the reaction conditions, protection of the amino ter-

minus of the amino acid is required. After the monomer is coupled to the end of the peptide, the N-terminal protecting group is removed, and another amino acid is coupled to the chain. This cycle of coupling and deprotecting is continued for each amino acid in the peptide sequence. See Merrifield, *J. Am. Chem. Soc.* (1963) 85:2149, and Atherton et al., "Solid Phase Peptide Synthesis" 1989, IRL Press, London, both incorporated herein by reference for all purposes. As described above, the use of a photoremovable protecting group allows removal of selected portions of the substrate surface, via patterned irradiation, during the deprotection cycle of the solid phase synthesis. This selectively allows spatial control of the synthesis—the next amino acid is coupled only to the irradiated areas.

In one embodiment, the photoremovable protecting groups of the present invention are attached to an activated ester of an amino acid at the amino terminus:



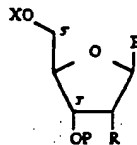
where R is the side chain of a natural or unnatural amino acid, X is a photoremovable protecting group, and Y is an activated carboxylic acid derivative. The photoremovable protecting group, X, is preferably NVOC, NPOC, PyROC, MeNVOC, MeNPOC, and the like as discussed above. The activated ester, Y, is preferably a reactive derivative having a high coupling efficiency, such as an acyl halide, mixed anhydride, N-hydroxysuccinimide ester, perfluorophenyl ester, or urethane protected acid, and the like. Other activated esters and reaction conditions are well known (See Atherton et al.).

2. Use of Photoremovable Groups During Solid-Phase Synthesis of Oligonucleotides

The formation of oligonucleotides on a solid-phase support requires the stepwise attachment of a nucleotide to a substrate-bound growing oligomer. In order to prevent unwanted polymerization of the monomeric nucleotide under the reaction conditions, protection of the 5'-hydroxyl group of the nucleotide is required. After the monomer is coupled to the end of the oligomer, the 5'-hydroxyl protecting group is removed, and another nucleotide is coupled to the chain. This cycle of coupling and deprotecting is continued for each nucleotide in the oligomer sequence. See Gait, "Oligonucleotide Synthesis: A Practical Approach" 1984, IRL Press, London, incorporated herein by reference for all purposes. As described above, the use of a photoremovable protecting group allows removal, via patterned irradiation, of selected portions of the substrate surface during the deprotection cycle of the solid phase synthesis. This selectively allows spatial control of the synthesis—the next nucleotide is coupled only to the irradiated areas.

Oligonucleotide synthesis generally involves coupling an activated phosphorous derivative on the 3'-hydroxyl group of a nucleotide with the 5'-hydroxyl group of an oligomer bound to a solid support. Two major chemical methods exist to perform this coupling: the phosphate-triester and phosphoramidite methods (See Gait). Protecting groups of the present invention are suitable for use in either method.

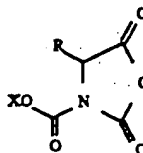
In a preferred embodiment, a photoremovable protecting group is attached to an activated nucleotide on the 5'-hydroxyl group:



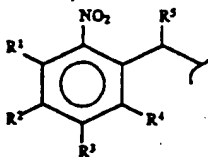
where B is the base attached to the sugar ring; R is a hydrogen atom when the sugar is deoxyribose or R is a hydroxyl group when the sugar is ribose; P represents an activated phosphorous group; and X is a photoremovable protecting group. The photoremovable protecting group, X, is preferably NV, NP, PyR, MeNV, MeNP, and the like as described above. The activated phosphorous group, P, is preferably a reactive derivative having a high coupling efficiency, such as a phosphate-triester, phosphoramidite or the like. Other activated phosphorous derivatives, as well as reaction conditions, are well known (See Gait).

E. Amino Acid N-Carboxy Anhydrides Protected With a Photoremovable Group

During Merrifield peptide synthesis, an activated ester of one amino acid is coupled with the free amino terminus of a substrate-bound oligomer. Activated esters of amino acids suitable for the solid phase synthesis include halo-formate, mixed anhydride, imidazolyl formate, acyl halide, and also includes formation of the activated ester in situ and the use of common reagents such as DCC and the like (See Atherton et al.). A preferred protected and activated amino acid has the general formula:



where R is the side chain of the amino acid and X is a photoremovable protecting group. This compound is a urethane-protected amino acid having a photoremovable protecting group attached to the amine. A more preferred activated amino acid is formed when the photoremovable protecting group has the general formula:

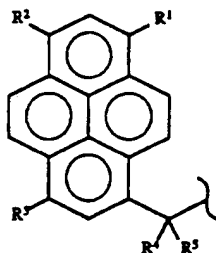


where R¹, R², R³, and R⁴ independently are a hydrogen atom, a lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxy, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido group, or adjacent substituents (i.e., R¹-R², R²-R³, R³-R⁴) are substituted oxygen groups that together form a cyclic acetal or ketal; and R⁵ is a hydrogen atom, an alkoxy, alkyl, hydrogen, halo, aryl, or alkenyl group.

A preferred activated amino acid is formed when the photoremovable protecting group is 6-nitroveratryloxycarbonyl. That is, R¹ and R⁴ are each a hydrogen atom, R² and R³ are each a methoxy group, and R⁵ is a hydrogen atom. Another preferred activated amino acid is formed when the photoremovable group is

6-nitropiperonyl: R^1 and R^4 are each a hydrogen atom. R^2 and R^3 together form a methylene acetal, and R^5 is a hydrogen atom. Other protecting groups are possible. Another preferred activated ester is formed when the photoremovable protecting group is methyl-6-nitroveratryl or methyl-6-nitropiperonyl.

Another preferred activated amino acid is formed when the photoremovable protecting group has the general formula:



where R^1 , R^2 , and R^3 independently are a hydrogen atom, a lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxy, thiol, thioether, amino, nitro, carboxyl, formate, formamido, sulfonates, sulfido or phosphido group, and R^4 and R^5 independently are a hydrogen atom, an alkoxy, alkyl, halo, aryl, hydrogen, or alkenyl group. The resulting compound is a urethane-protected amino acid having a pyrenylmethoxycarbonyl protecting group attached to the amine. A more preferred embodiment is formed when R^1 through R^5 are each a hydrogen atom.

The urethane-protected amino acids having a photoremovable protecting group of the present invention are prepared by condensation of an N-protected amino acid with an acylating agent such as an acyl halide, anhydride, chloroformate and the like (See Fuller et al., U.S. Pat. No. 4,946,942 and Fuller et al., *J. Amer. Chem. Soc.* (1990) 112:7414-7416, both herein incorporated by reference for all purposes).

Urethane-protected amino acids having photoremovable protecting groups are generally useful as reagents during solid-phase peptide synthesis, and because of the spatial selectivity possible with the photoremovable protecting group, are especially useful for the spatially addressable peptide synthesis. These amino acids are difunctional: the urethane group first serves to activate the carboxy terminus for reaction with the amine bound to the surface and, once the peptide bond is formed, the photoremovable protecting group protects the newly formed amino terminus from further reaction. These amino acids are also highly reactive to nucleophiles, such as deprotected amines on the surface of the solid support, and due to this high reactivity, the solid-phase peptide coupling times are significantly reduced, and yields are typically higher.

IV. Data Collection

A. Data Collection System

Substrates prepared in accordance with the above description are used in one embodiment to determine which of the plurality of sequences thereon bind to a receptor of interest. FIG. 11 illustrates one embodiment of a device used to detect regions of a substrate which contain fluorescent markers. This device would be used, for example, to detect the presence or absence of a labeled receptor such as an antibody which has bound to a synthesized polymer on a substrate.

Light is directed at the substrate from a light source 1002 such as a laser light source of the type well known to those

of skill in the art such as a model no. 2025 made by Spectra Physics. Light from the source is directed at a lens 1004 which is preferably a cylindrical lens of the type well known to those of skill in the art. The resulting output from the lens 1004 is a linear beam rather than a spot of light, resulting in the capability to detect data substantially simultaneously along a linear array of pixels rather than on a pixel-by-pixel basis. It will be understood that a cylindrical lens is used herein as an illustration of one technique for generating a linear beam of light on a surface, but that other techniques could also be utilized.

The beam from the cylindrical lens is passed through a dichroic mirror or prism (1006) and directed at the surface of the suitably prepared substrate 1008. Substrate 1008 is placed on an x-y translation stage 1009 such as a model no. PM500-8 made by Newport. Light at certain locations on the substrate will be fluoresced and transmitted along the path indicated by dashed lines back through the dichroic mirror, and focused with a suitable lens 1010 such as an f/1.4 camera lens on a linear detector 1012 via a variable f stop focusing lens 1014. Through use of a linear light beam, it becomes possible to generate data over a line of pixels (such as about 1 cm) along the substrate, rather than from individual points on the substrate. In alternative embodiments, light is directed at a 2-dimensional area of the substrate and fluoresced light detected by a 2-dimensional CCD array. Linear detection is preferred because substantially higher power densities are obtained.

Detector 1012 detects the amount of light fluoresced from the substrate as a function of position. According to one embodiment the detector is a linear CCD array of the type commonly known to those of skill in the art. The x-y translation stage, the light source, and the detector 1012 are all operably connected to a computer 1016 such as an IBM PC-AT or equivalent for control of the device and data collection from the CCD array.

In operation, the substrate is appropriately positioned by the translation stage. The light source is then illuminated, and intensity data are gathered with the computer via the detector.

FIG. 12 illustrates the architecture of the data collection system in greater detail. Operation of the system occurs under the direction of the photon counting program 1102 (photon), included herewith as Appendix B. The user inputs the scan dimensions, the number of pixels or data points in a region, and the scan speed to the counting program. Via a GPIB bus 1104 the program (in an IBM PC compatible computer, for example) interfaces with a multichannel scaler 1106 such as a Stanford Research SR 430 and an x-y stage controller 1108 such as a PM500. The signal from the light from the fluorescing substrate enters a photon counter 1110, providing output to the scaler 1106. Data are output from the scaler indicative of the number of counts in a given region. After scanning a selected area, the stage controller is activated with commands for acceleration and velocity, which in turn drives the scan stage 1112 such as a PM500-A to another region.

Data are collected in an image data file 1114 and processed in a scaling program 1116, also included in Appendix B. A scaled image is output for display on, for example, a VGA display 1118. The image is scaled based on an input of the percentage of pixels to clip and the minimum and maximum pixel levels to be viewed. The system outputs for use the min and max pixel levels in the raw data.

B. Data Analysis

The output from the data collection system is an array of data indicative of fluorescent intensity versus location on the substrate. The data are typically taken over regions substan-

tially smaller than the area in which synthesis of a given polymer has taken place. Merely by way of example, if polymers were synthesized in squares on the substrate having dimensions of 500 microns by 500 microns, the data may be taken over regions having dimensions of 5 microns by 5 microns. In most preferred embodiments, the regions over which fluorescence data are taken across the substrate are less than about $\frac{1}{4}$ the area of the regions in which individual polymers are synthesized, preferably less than $\frac{1}{10}$ the area in which a single polymer is synthesized, and most preferably less than $\frac{1}{100}$ the area in which a single polymer is synthesized. Hence, within any area in which a given polymer has been synthesized, a large number of fluorescence data points are collected.

A plot of number of pixels versus intensity for a scan of a cell when it has been exposed to, for example, a labeled antibody will typically take the form of a bell curve, but spurious data are observed, particularly at higher intensities. Since it is desirable to use an average of fluorescent intensity over a given synthesis region in determining relative binding affinity, these spurious data will tend to undesirably skew the data.

Accordingly, in one embodiment of the invention the data are corrected for removal of these spurious data points, and an average of the data points is thereafter utilized in determining relative binding efficiency.

FIG. 13 illustrates one embodiment of a system for removal of spurious data from a set of fluorescence data such as data used in affinity screening studies. A user or the system inputs data relating to the chip location and cell corners at step 1302. From this information and the image file, the system creates a computer representation of a histogram at step 1304, the histogram (at least in the form of a computer file) plotting number of data pixels versus intensity.

For each cell, a main data analysis loop is then performed. For each cell, at step 1306, the system calculates the total intensity or number of pixels for the bandwidth centered around varying intensity levels. For example, as shown in the plot to the right of step 1306, the system calculates the number of pixels within the band of width w . The system then "moves" this bandwidth to a higher center intensity, and again calculates the number of pixels in the bandwidth. This process is repeated until the entire range of intensities has been scanned, and at step 1308 the system determines which band has the highest total number of pixels. The data within this bandwidth are used for further analysis. Assuming the bandwidth is selected to be reasonably small, this procedure will have the effect of eliminating spurious data located at the higher intensity levels. The system then repeats at step 1310 if all cells have been evaluated, or repeats for the next cell.

At step 1312 the system then integrates the data within the bandwidth for each of the selected cells, sorts the data at step 1314 using the synthesis procedure file, and displays the data to a user on, for example, a video display or a printer.

V. Representative Applications

A. Oligonucleotide Synthesis

The generality of light directed spatially addressable parallel chemical synthesis is demonstrated by application to nucleic acid synthesis.

1. Example

Light activated formation of a thymidinecytidine dimer was carried out. A three dimensional representation of a fluorescence scan showing a checkerboard pattern generated by the light-directed synthesis of a dinucleotide is shown in FIG. 8. 5'-nitroveratryl thymidine was attached to a synthesis substrate through the 3' hydroxyl group. The nitroveratryl protecting groups were removed by illumination through a 500 mm checkerboard mask. The substrate was then treated with phosphoramidite activated 2'-deoxycytidine. In order to follow the reaction fluorometrically, the deoxycytidine had been modified with an Fmoc protected aminohexyl linker attached to the exocyclic amine (5'-O-dimethoxytrityl-4-N-(6-N-fluorenylmethylcarbamoyl-hexylcarboxy)-2'-deoxycytidine). After removal of the Fmoc protecting group with base, the regions which contained the dinucleotide were fluorescently labelled by treatment of the substrate with 1 mM FITC in DMF for one hour.

The three-dimensional representation of the fluorescent intensity data in FIG. 14 clearly reproduces the checkerboard illumination pattern used during photolysis of the substrate. This result demonstrates that oligonucleotides as well as peptides can be synthesized by the light-directed method.

VI. Conclusion

The inventions herein provide a new approach for the simultaneous synthesis of a large number of compounds. The method can be applied whenever one has chemical building blocks that can be coupled in a solid-phase format, and when light can be used to generate a reactive group.

The above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this disclosure. Merely by way of example, while the invention is illustrated primarily with regard to peptide and nucleotide synthesis, the invention is not so limited. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1.1) NUMBER OF SEQUENCES: 21

(2) INFORMATION FOR SEQ ID NO:1:

(2.1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Gly Gly Phe Leu
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Gly Gly Phe Leu
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Gly Ala Gly Phe
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Tyr Gly Ala Phe Leu Ser
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Gly Ala Phe Ser
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: peptide

(2) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Gly Ala Phe Leu
 1 5

(2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: peptide

(2) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Tyr Gly Gly Phe Leu Ser
 1 5

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: peptide

(2) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr Gly Ala Phe
 1

(2) INFORMATION FOR SEQ ID NO:9:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: peptide

(2) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Gly Ala Leu Ser
 1 5

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: peptide

(2) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Tyr Gly Gly Phe Ser
 1 5

(2) INFORMATION FOR SEQ ID NO:11:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Tyr Gly Ala Leu

1

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Tyr Gly Ala Phe Leu Phe

1

5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Tyr Gly Ala Phe Phe

1

5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Tyr Gly Gly Leu Ser

1

5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Gly Gly Phe Leu

1

5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Tyr Gly Ala Phe Ser Phe
 1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Gly Ala Phe Leu Ser Phe
 1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Tyr Gly Ala Phe Met Gln
 1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Tyr Gly Ala Phe Met
 1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Tyr Gly Ala Phe Gln
 1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

H

This Page Blank (uspto)



US005837832A

United States Patent [19]

Chee et al.

[11] Patent Number: 5,837,832

[45] Date of Patent: Nov. 17, 1998

[54] ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS

[75] Inventors: Mark Chee, Palo Alto; Maureen T. Cronin, Los Altos; Stephen P. A. Fodor, Palo Alto; Xiaohua X. Huang; Earl A. Hubbell, both of Mt. View; Robert J. Lipshutz; Peter E. Lobban, both of Palo Alto; MacDonald S. Morris, San Jose; Edward L. Sheldon, Menlo Park, all of Calif.

[73] Assignee: Affymetrix, Inc., Santa Clara, Calif.

[21] Appl. No.: 441,887

[22] Filed: May 16, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 143,312, Oct. 26, 1993, abandoned, which is a continuation-in-part of Ser. No. 82,937, Jun. 25, 1993, abandoned.

[51] Int. Cl.⁶ C12Q 1/68

[52] U.S. Cl. 536/22.1; 435/6; 435/91.1; 436/501; 536/23.1; 536/24.1; 536/24.3; 536/24.31; 536/24.32; 536/24.33; 536/25.3; 422/68.1; 935/77; 935/78; 935/88

[58] Field of Search 435/6, 91.1, 810; 436/501; 536/22.1, 23.1, 24.1, 24.3-24.33, 25, 3; 935/77, 78, 88; 422/68.1

[56] References Cited

U.S. PATENT DOCUMENTS

4,656,127 4/1987 Mundy 435/6
4,683,195 7/1987 Mallis et al. 435/6
5,002,867 3/1991 Macevitz 435/6
5,143,854 9/1992 Pirrung et al. 436/518
5,202,231 4/1993 Drmanac et al. 435/6
5,273,632 12/1993 Stockham et al. 204/180.1
5,527,681 6/1996 Homes 435/6

FOREIGN PATENT DOCUMENTS

WO 89/10977 11/1989 WIPO C12Q 1/68

WO 89/11548 11/1989 WIPO C12Q 1/68
WO 90/00626 1/1990 WIPO C12Q 1/68
WO 90/03382 4/1990 WIPO C07H 21/00
WO 92/10092 6/1992 WIPO A01N 1/02
WO 92/10588 6/1992 WIPO C12Q 1/68
WO 93/10588 6/1992 WIPO C12Q 1/68
WO 93/17126 9/1993 WIPO C12Q 1/68

OTHER PUBLICATIONS

Maram et al. (1980) *Methods in Enzymology*, vol. 65, pp. 449-559.

Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1989, pp. 1145-1147

Stratagene 1988 Catalog, pp. 39.

Elder, J.K., "Analysis of DNA oligonucleotide hybridization data by maximum entropy," *Maximum Entropy and Bayesian Methods*, pp. 1-10, Paris (1992).

Lipshutz, Robert J., "Likelihood DNA sequencing by hybridization," *J. of Biomolecular Structure & Dynamics* 11:637-653 (1993).

Ying Luo et al., "Cellular protein modulates effects of human immunodeficiency virus type 1 rev," *J. of Virology* 68:3850-3856 (1994).

Querat et al., "Nucleotide sequence analysis of SA-OMV, a Visna-related ovine lentivirus: phylogenetic history of lentiviruses," *Virology* 175:434-447 (1990).

Ratner et al., "Complete nucleotide sequence of the AIDS virus, HTLV-III," *Nature* 313:277-284 (1985).

(List continued on next page.)

Primary Examiner—Ardin H. Marschel

Attorney, Agent, or Firm—Townsend & Townsend & Crew

[57] ABSTRACT

DNA chips containing arrays of oligonucleotide probes can be used to determine whether a target nucleic acid has a nucleotide sequence identical to or different from a specific reference sequence. The array of probes comprises probes exactly complementary to the reference sequence, as well as probes that differ by one or more bases from the exactly complementary probes.

18 Claims, 40 Drawing Sheets

ATTTTCATTCTGTATTG	Wild-Type Lane
X	A-Lane
X	C-Lane
X	G-Lane
X	T-Lane
	Blank Lane
CCGACTGCGAGTCGTTA	Wild-Type Lane
X	A-Lane
X	C-Lane
X	G-Lane
X	T-Lane
	Blank Lane



3' - CCGACTGCGAGTCGTT
3' - CCGACTACAGTCGTT
3' - CCGACTCCAGTCGTT
3' - CCGACTGCGAGTCGTT
3' - CCGACTTCAGTCGTT

OTHER PUBLICATIONS

Wain-Hobson et al., "Nucleotide sequence of the AIDS virus, LAV," *Cell* 40:9-17 (1985).

Southern et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation Using Experimental Models," *Genomics* (1992) 13:1008-1017.

Sanger et al., "DNA sequencing with chain-terminating inhibitors," *Proc. Natl. Acad. Sci. USA* (1977) 74:5463-5467.

M. Cronin et al., Hybridization to Arrays of Oligonucleotides, Poster Presentation: Nucleic Acids In Medical Applications Conference sponsored by AACC, Jan. 1993, published in conference syllabus, Cancun, Mexico.

M.S. Chee et al., Towards Sequencing Mitochondrial DNA Polymorphisms by Hybridization to a Custom Oligonucleotide Probe Array, American Society of Human Genetics 43rd Annual Meeting, Oct. 5-9, 1993, New Orleans, LA.

M.S. Chee et al., Genetic Analysis by Hybridization to Sequence-Specific DNA Arrays, Genome Sequencing and Analysis Conference V, Oct. 23-27, 1993, Hilton Head, SC.

P.E. Lobban et al., DNA Chips for Genetic Analysis, Genome Sequencing and Analysis Conference V, Oct. 23-27, 1993, Hilton Head, SC.

R. Lipshutz et al., Oligonucleotide Arrays for Hybridization Analysis, Genome Sequencing and Analysis Conference V, Oct. 23-27, 1993, Hilton Head, SC.

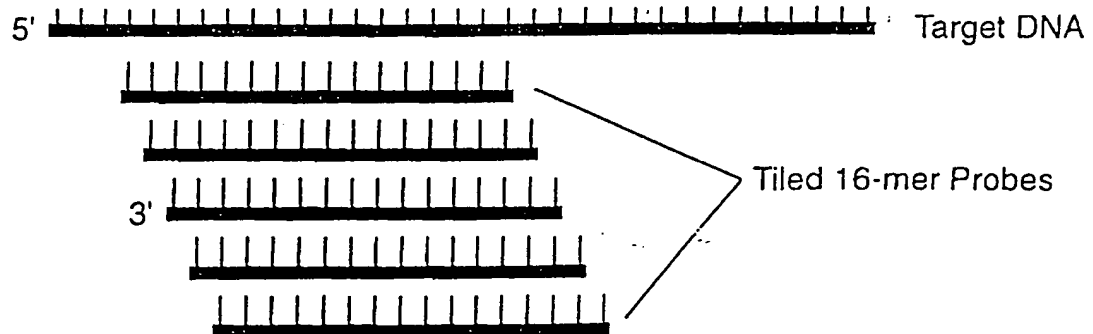
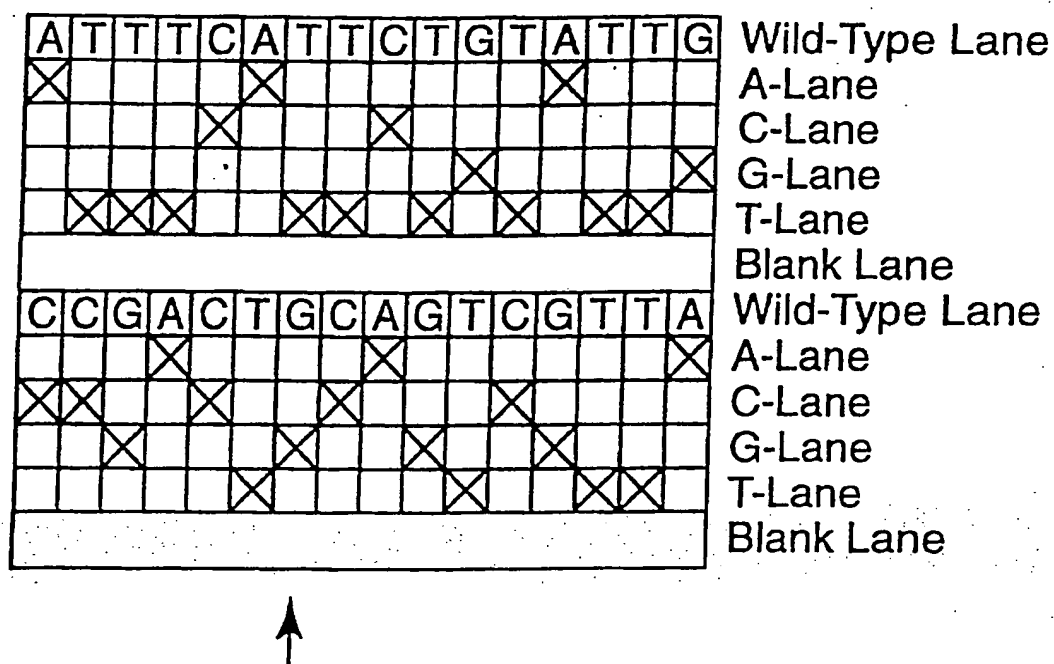


Fig. 1



3' - CCGACT**G**CAGTCGTT
3' - CCGACT**A**CAGTCGTT
3' - CCGACT**C**CAGTCGTT
3' - CCGACT**G**CAGTCGTT
3' - CCGACT**T**CAGTCGTT

Fig. 2

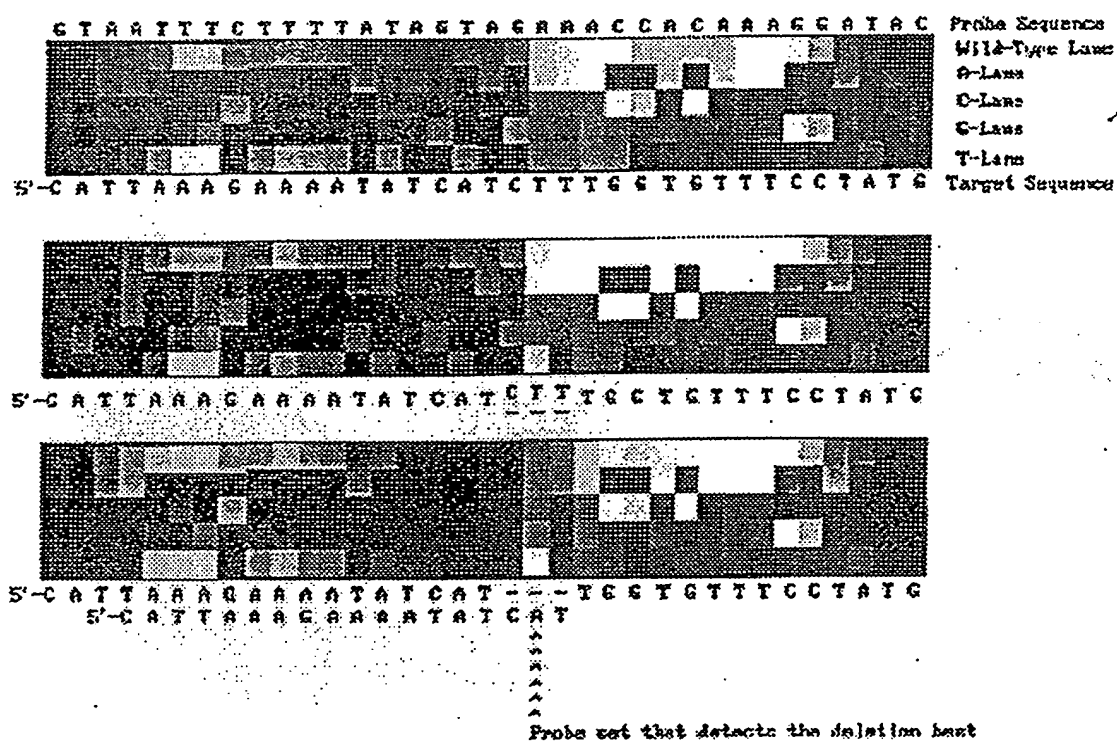


FIG. 3

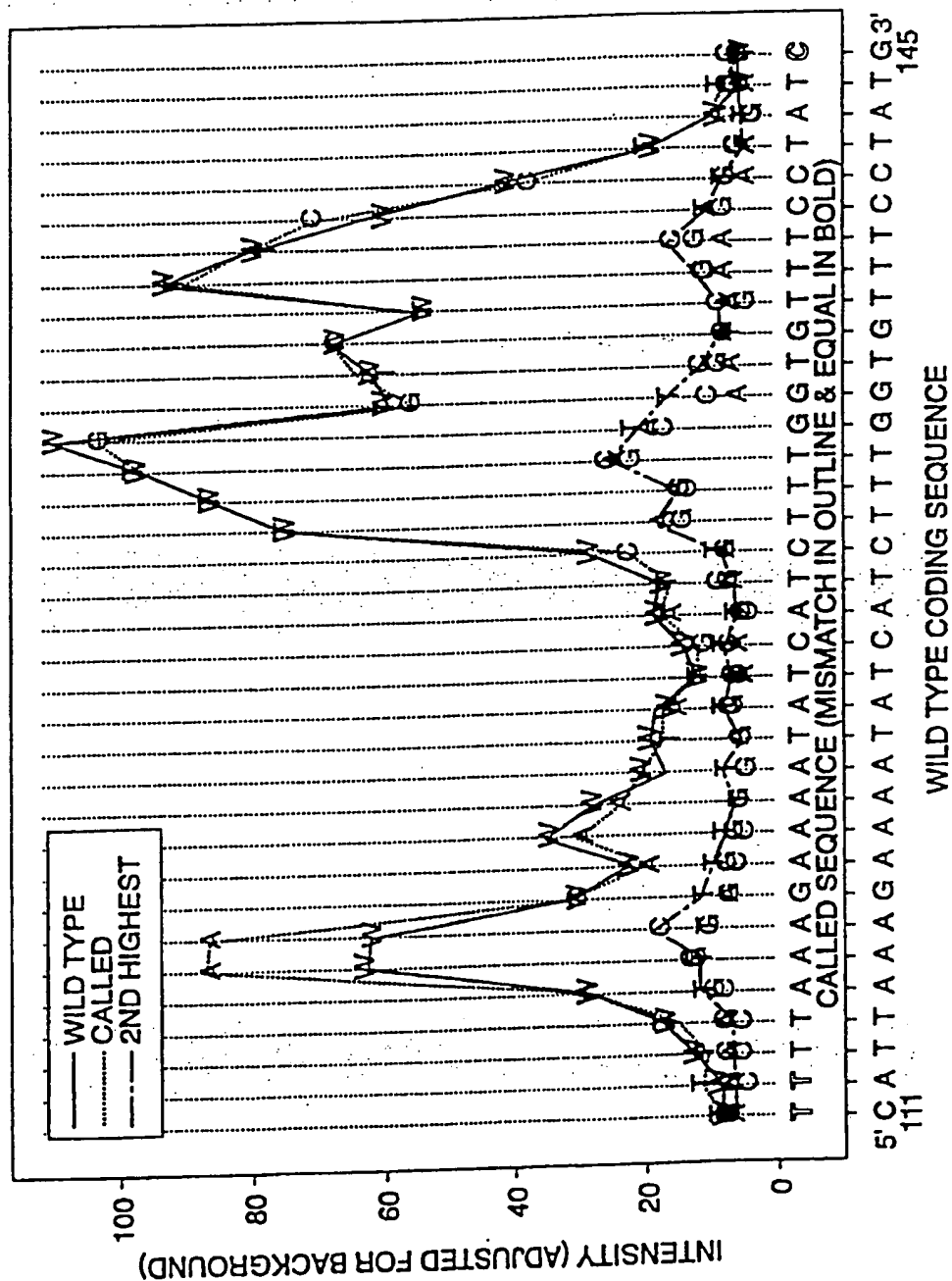


Fig. 4A

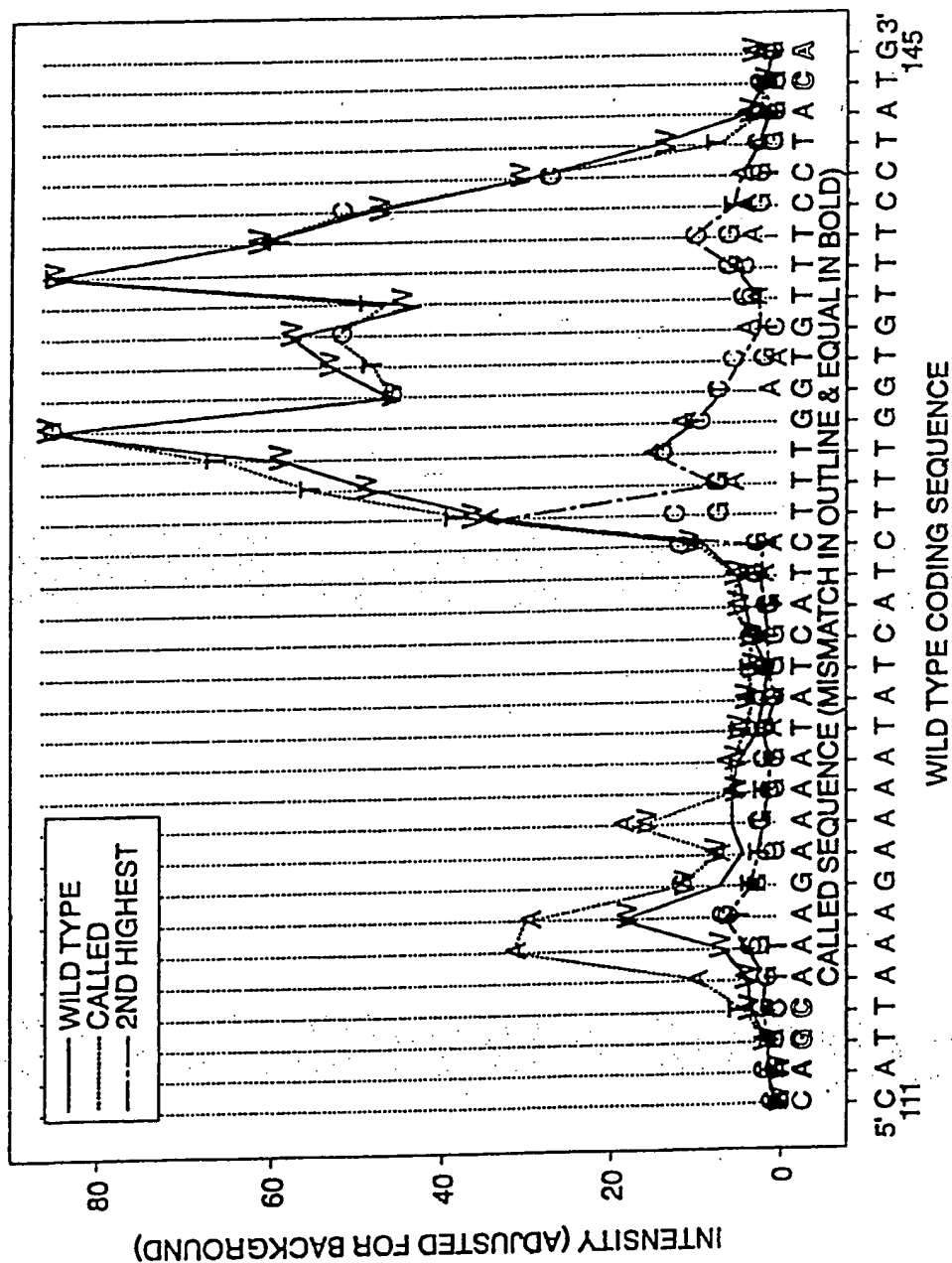


Fig. 4B

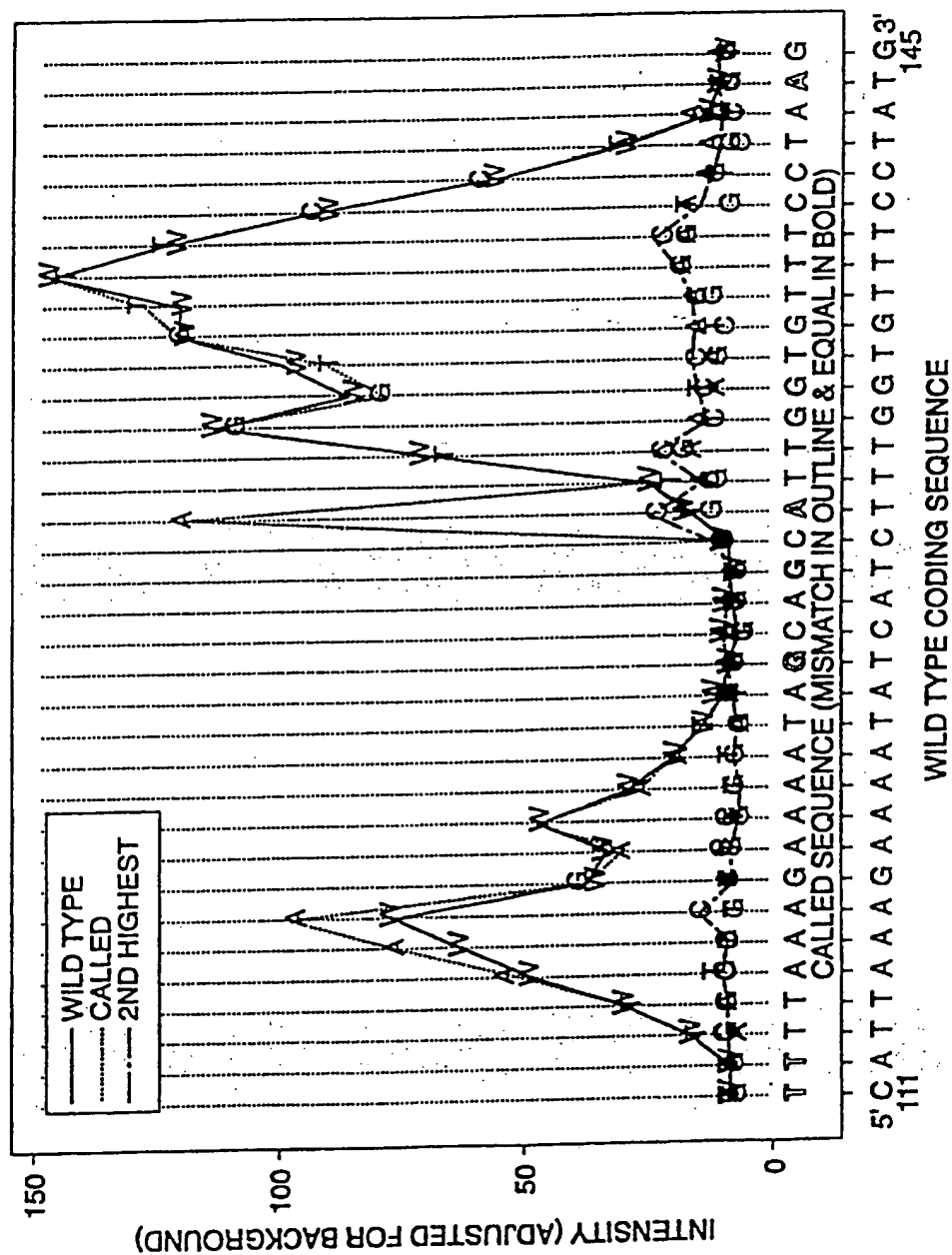


Fig. 4C

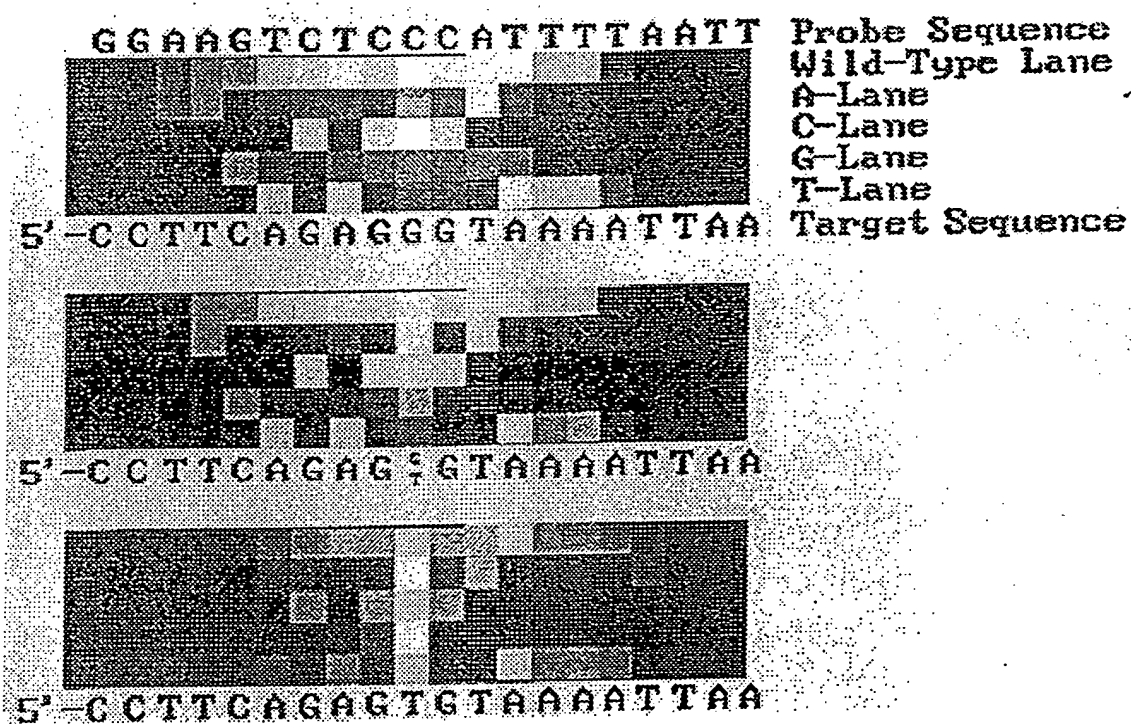


FIG. 5

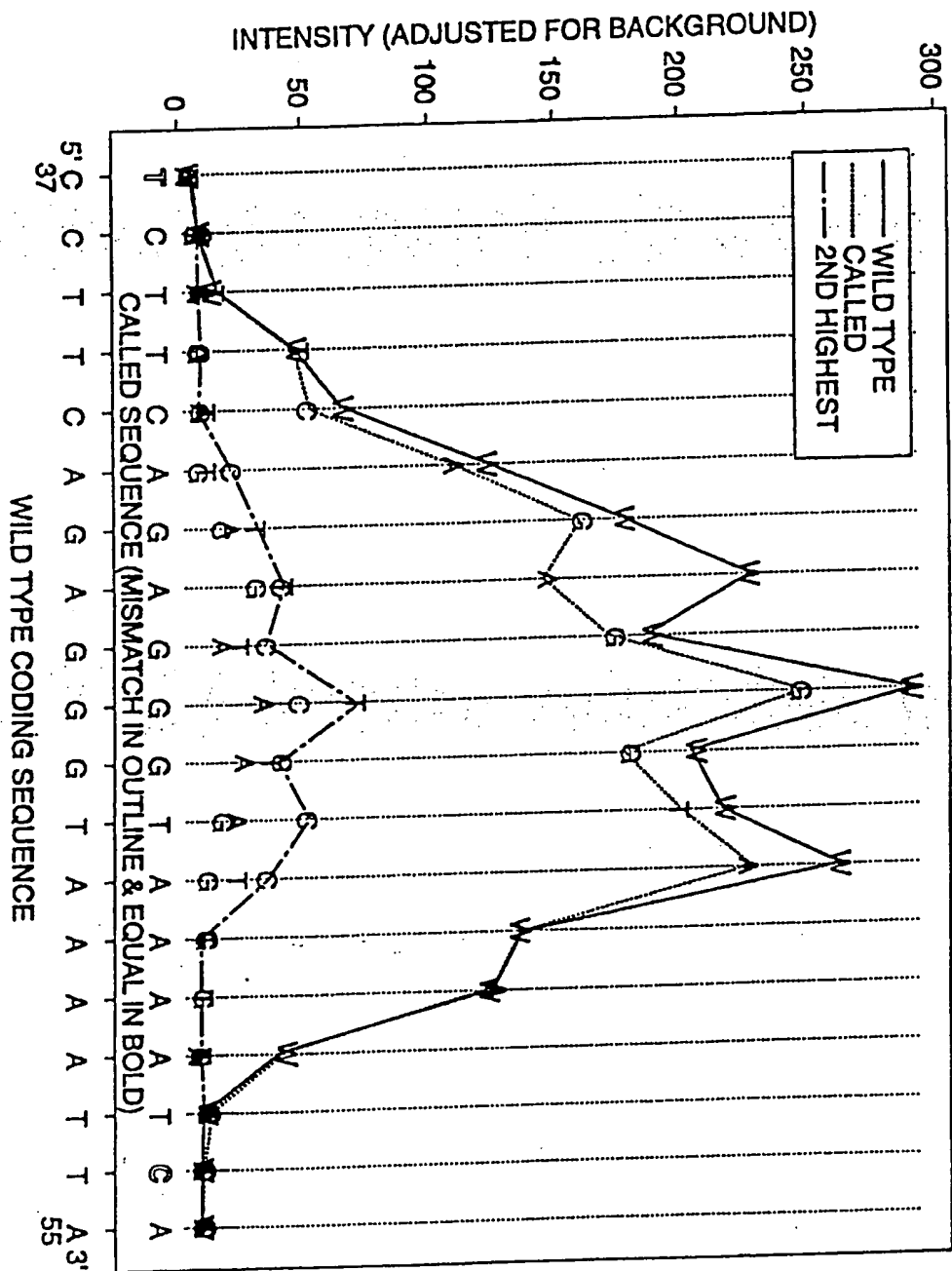


Fig. 6A

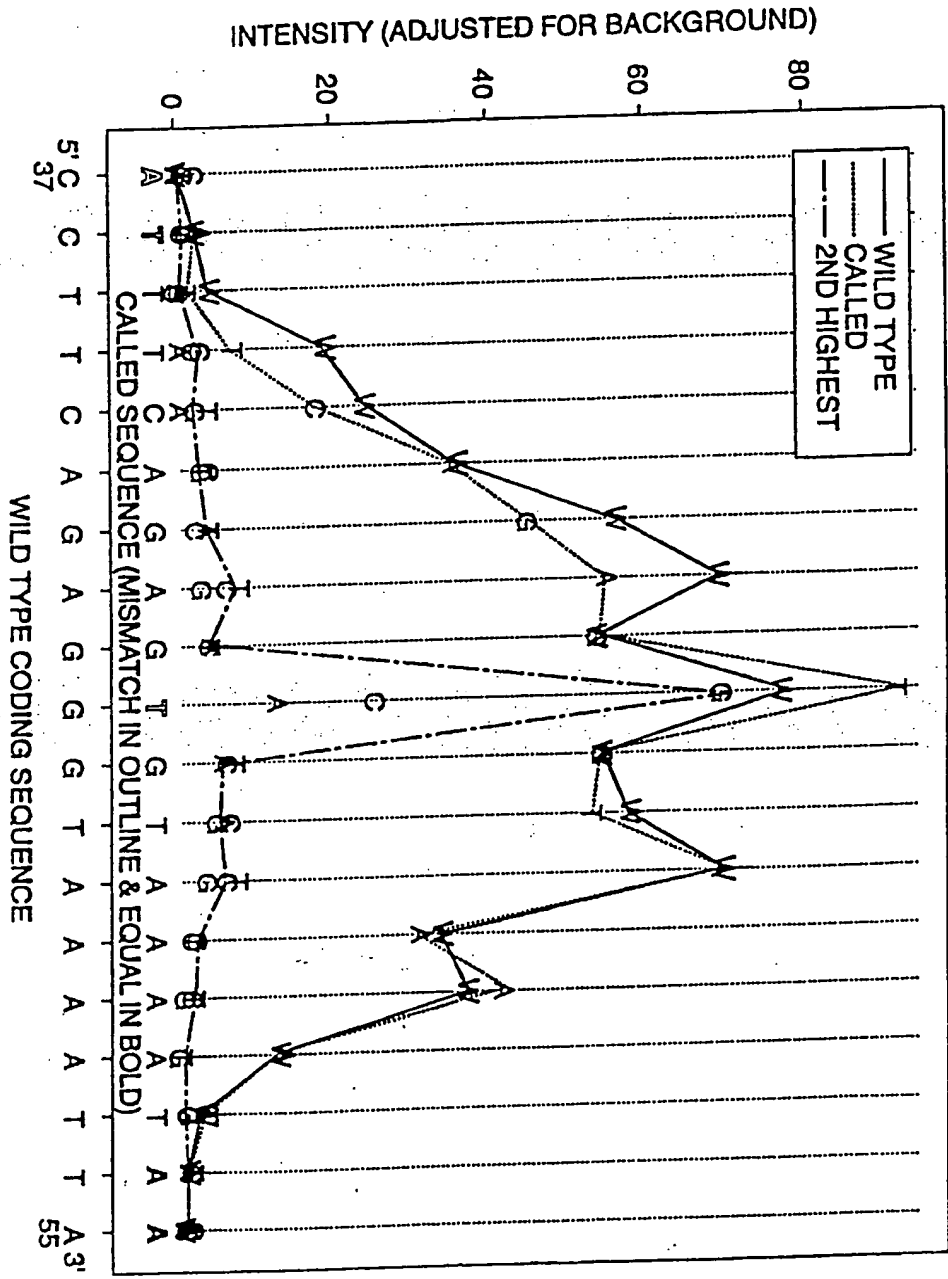


Fig. 6B

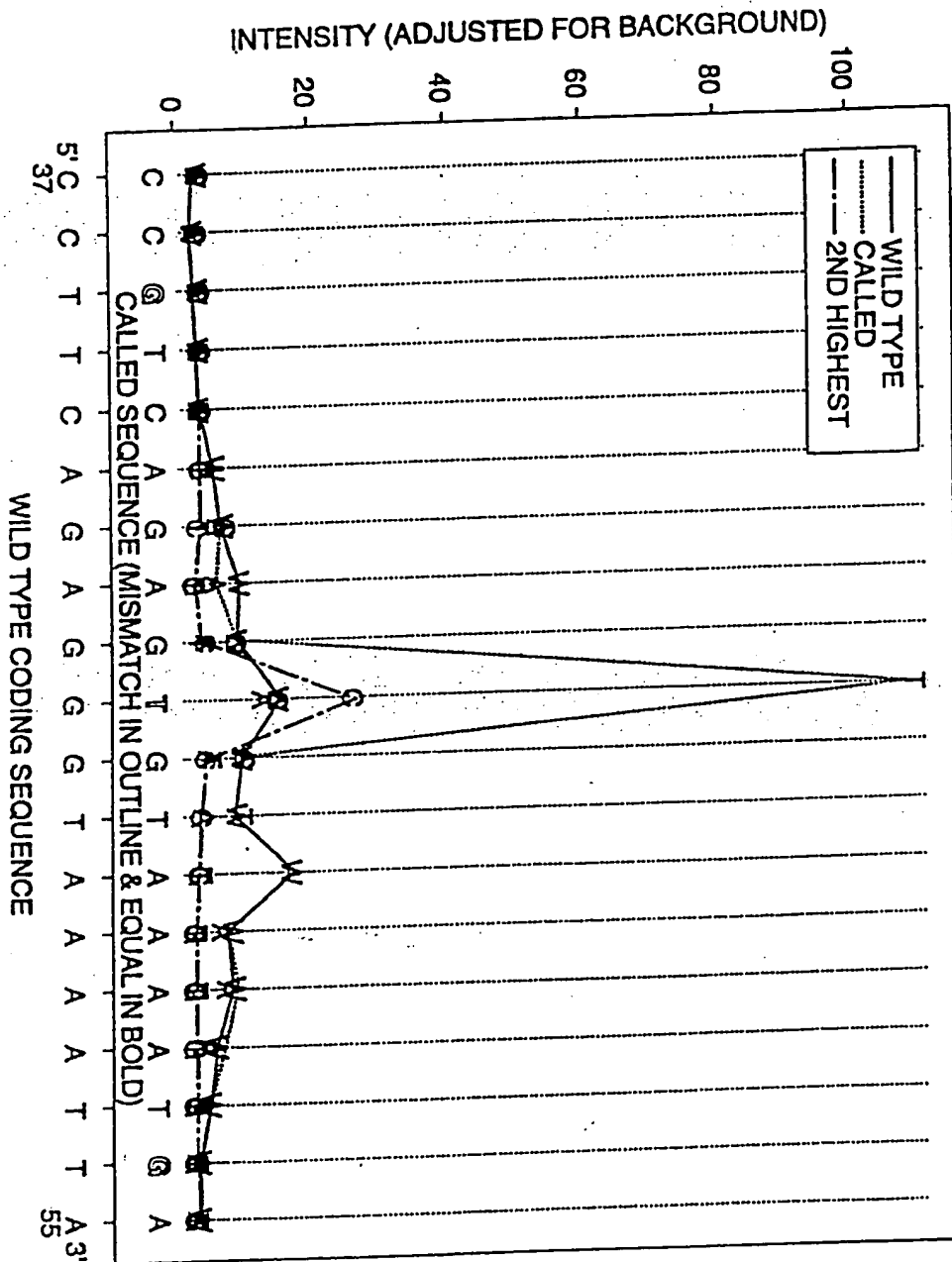


Fig. 6C

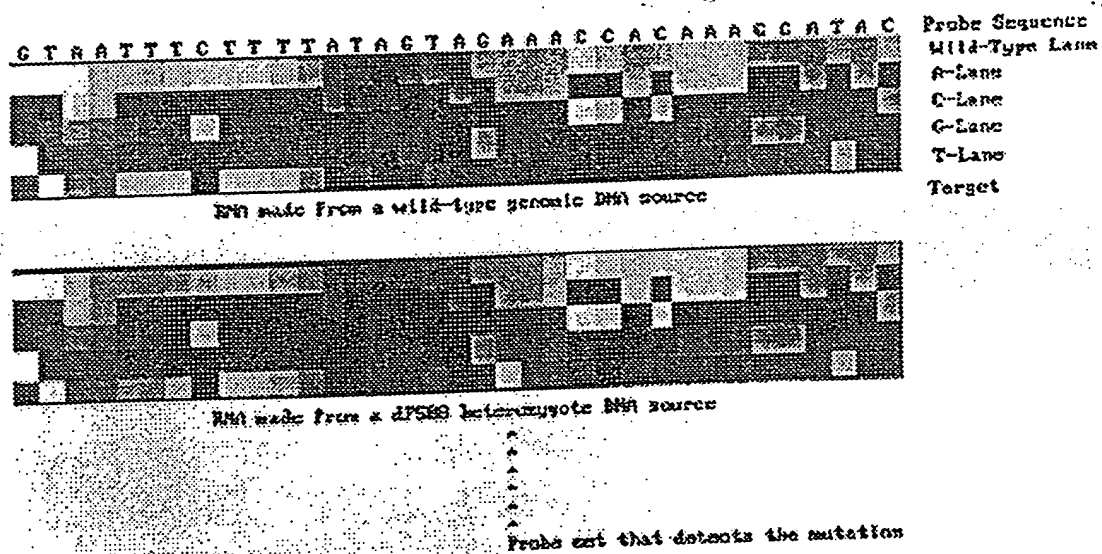


FIG. 7

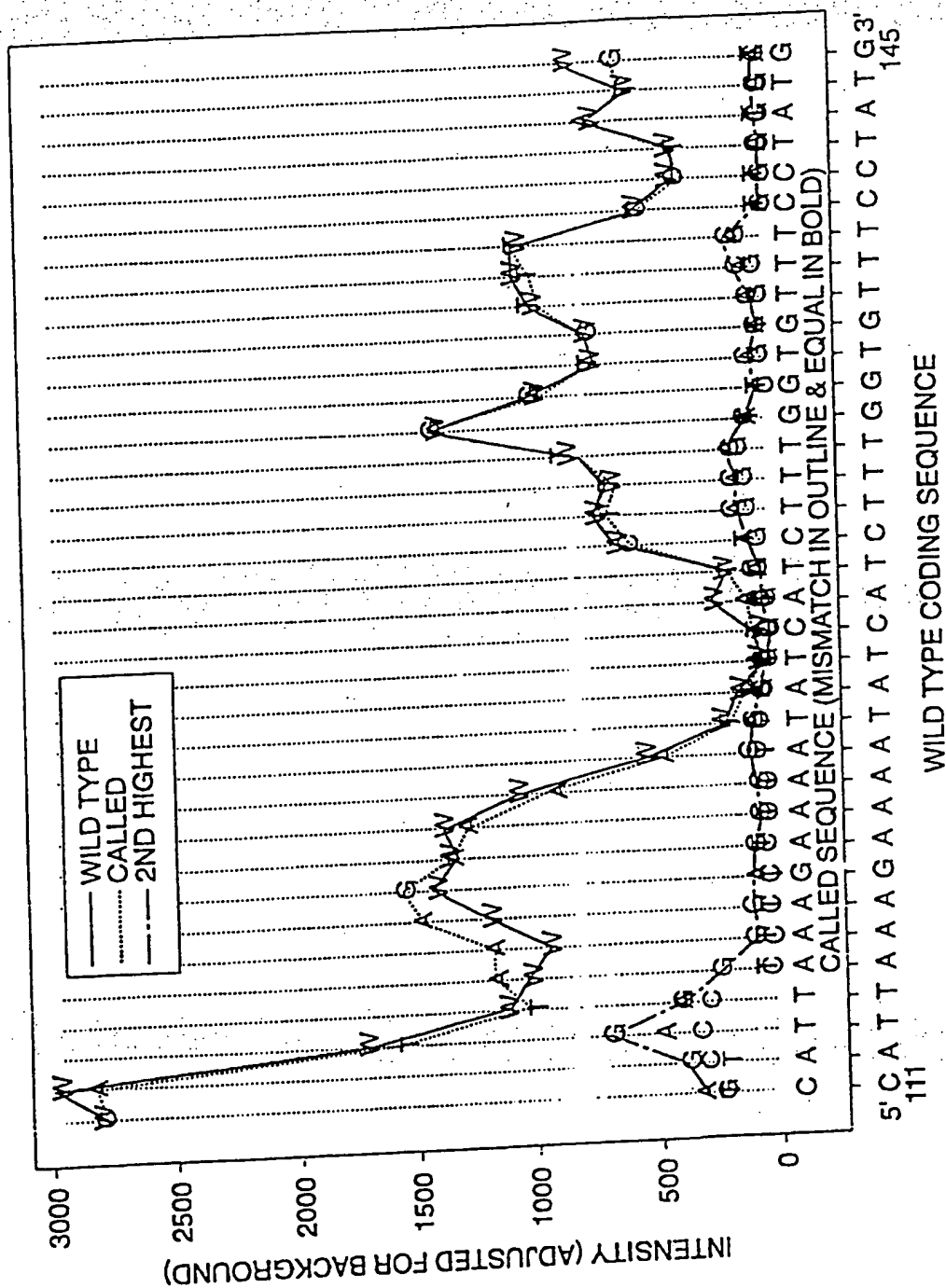


Fig. 8A

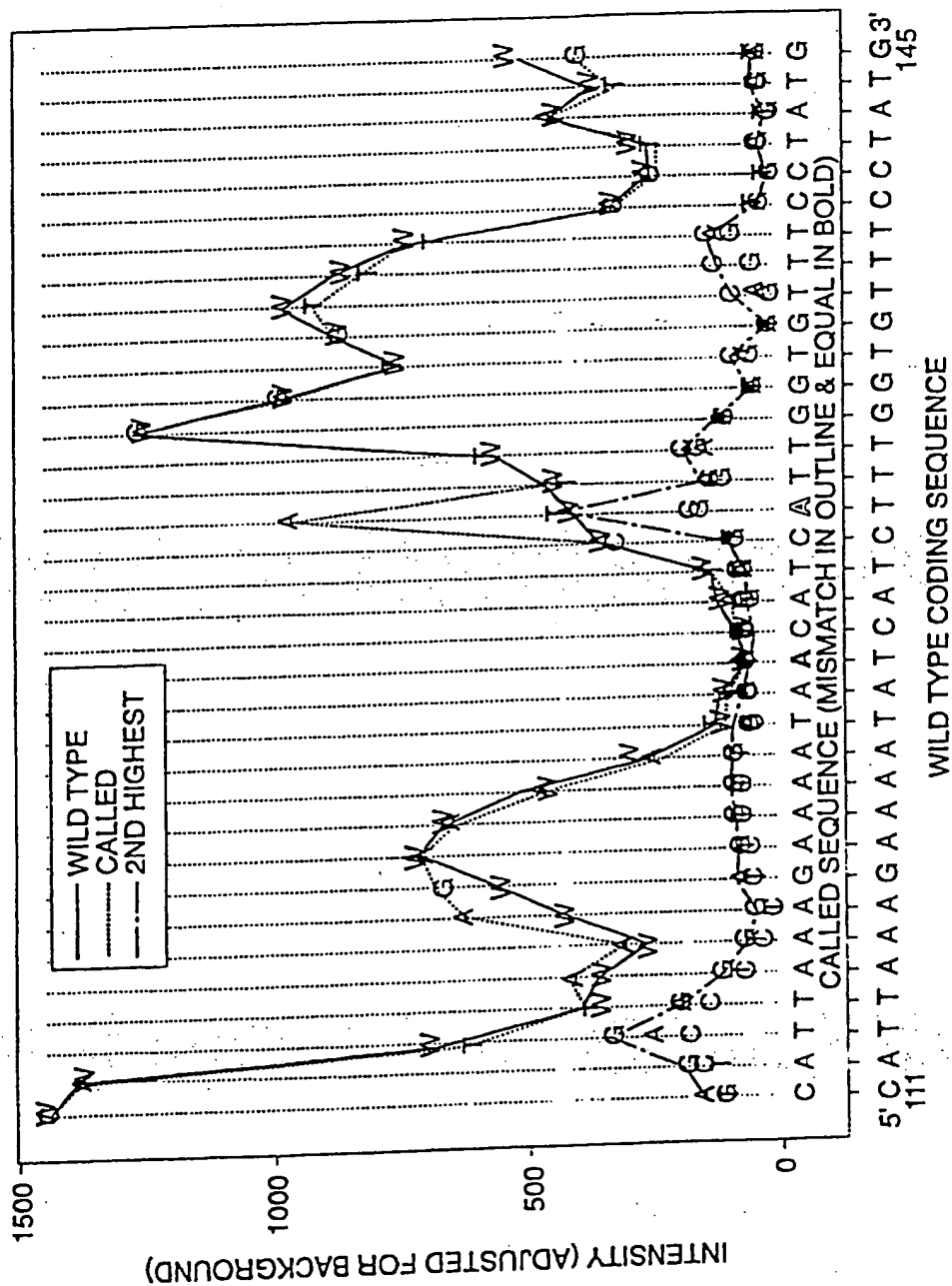


Fig. 8B

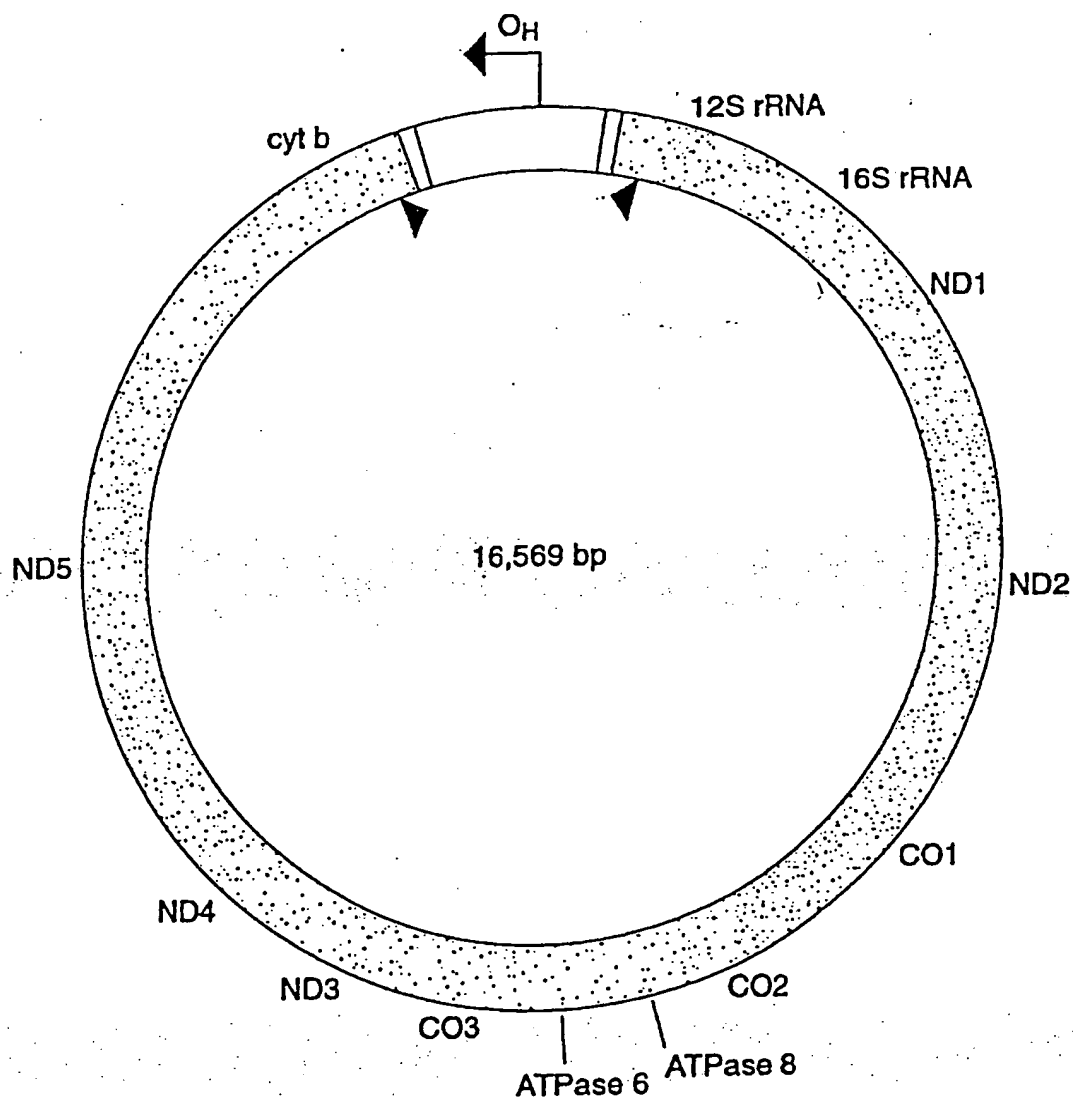
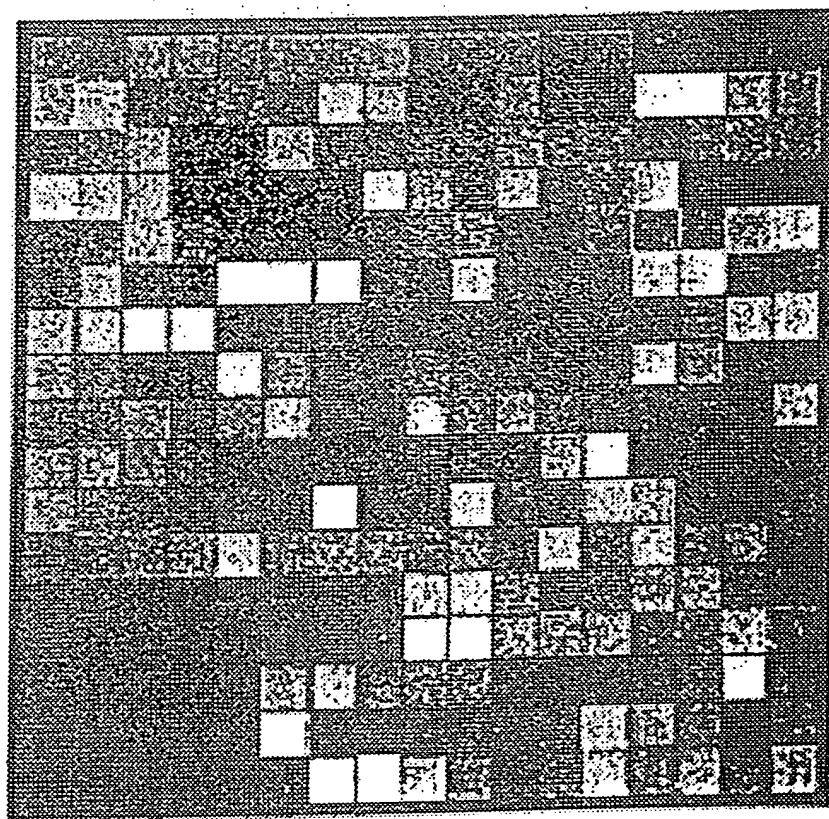
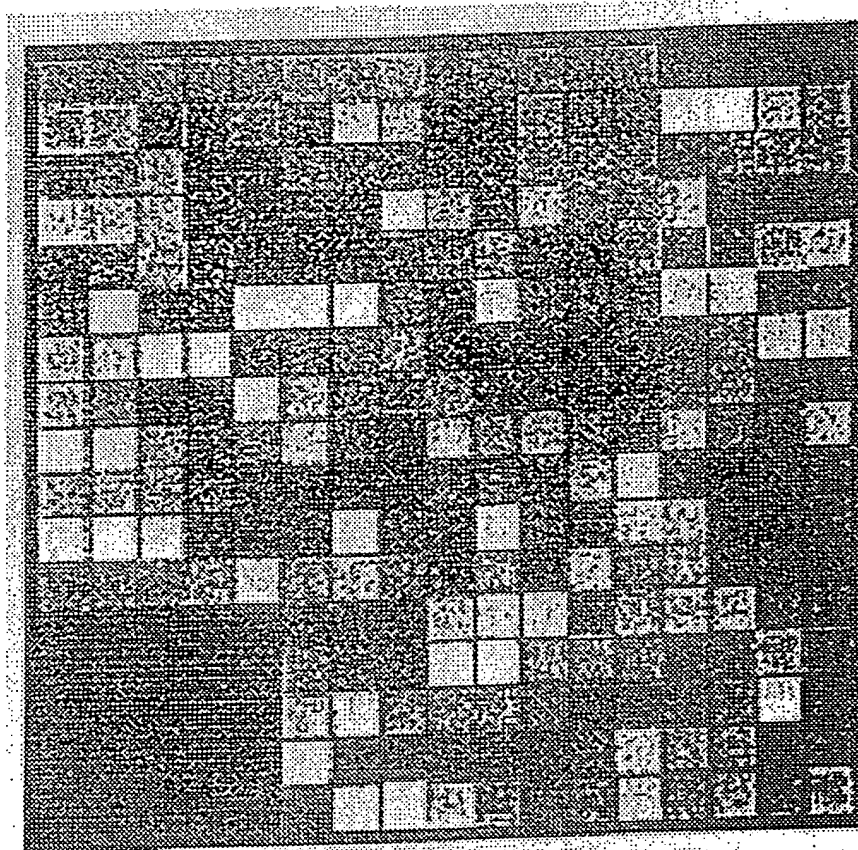


Fig. 9



mt4

FIG. 10



mt5

FIG. 11

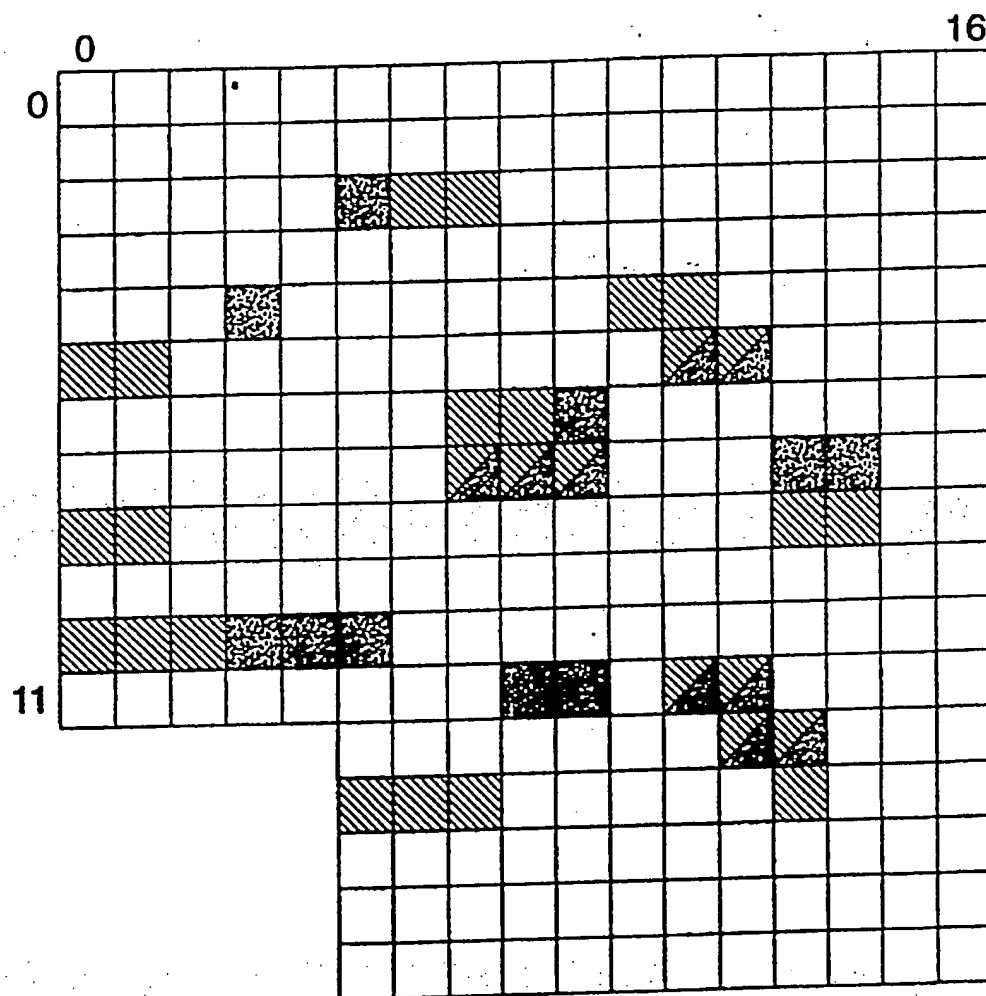


Fig. 12

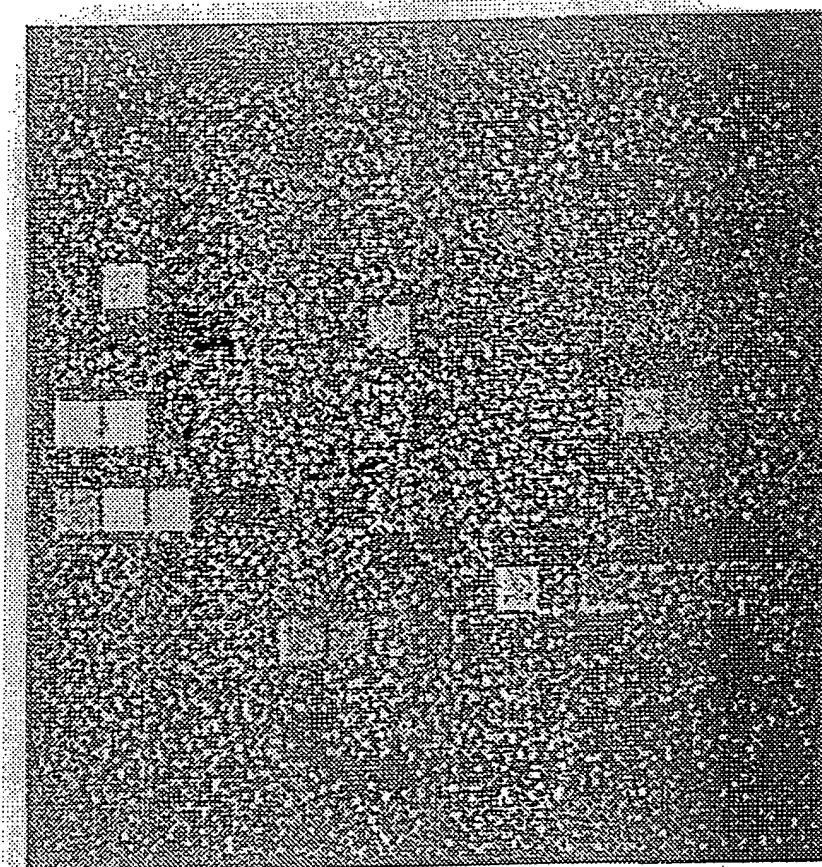


FIG. 13

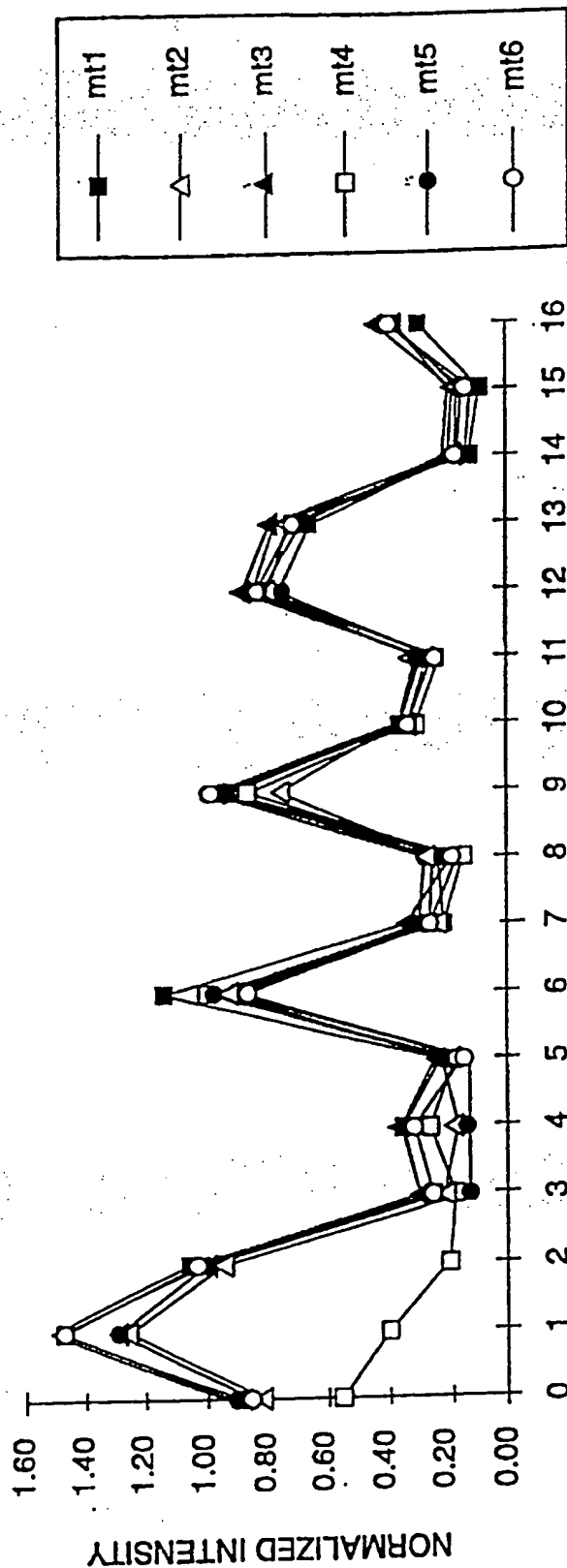
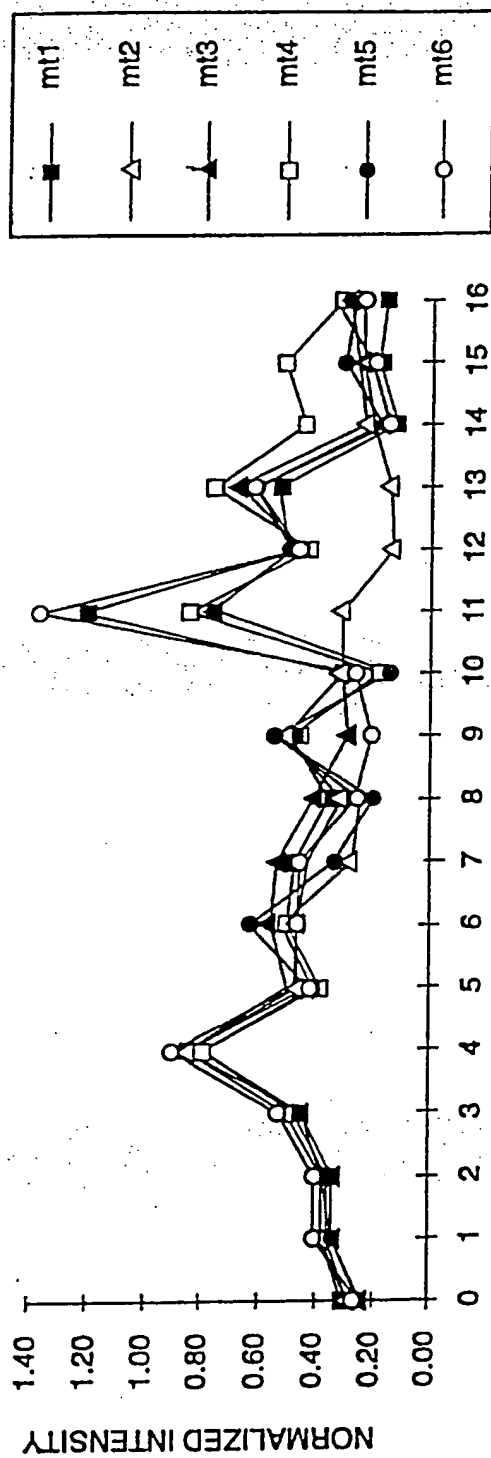


Fig. 14A

PROBE POSITION	0	1	2	3	4	5
PROBE LENGTH	13	13	12	12	12	12
SAMPLE (mt1 → 6)	4	4	4	2,5	2,5	2,5
MISMATCH POSITION FROM 3' OF PROBE	12	5	3	12	7	2
BASE CHANGE	t → a	t → a	t → a	t → c	t → c	t → c



PROBE POSITION IN ROW 11 OF ARRAY

PROBE POSITION	6	7	8	9	10	11	12	12
PROBE LENGTH	13	12	12	13	14	13	12	12
SAMPLE (mt1 → 6)	2	2, 5	2, 5, 6	3, 6	3, 4, 5	2, 4, 5	2	2
MISMATCH POSITION FROM 3' OF PROBE	13	9, 10	3, 4, 11	11, 5	4, 11, DOUBLE	11, 3, DOUBLE	6	3
BASE CHANGE	c → t	c → t	c → t t → c	t → c	t → c DOUBLE	g → a t → c DOUBLE	g → a	g → a

Fig. 14B

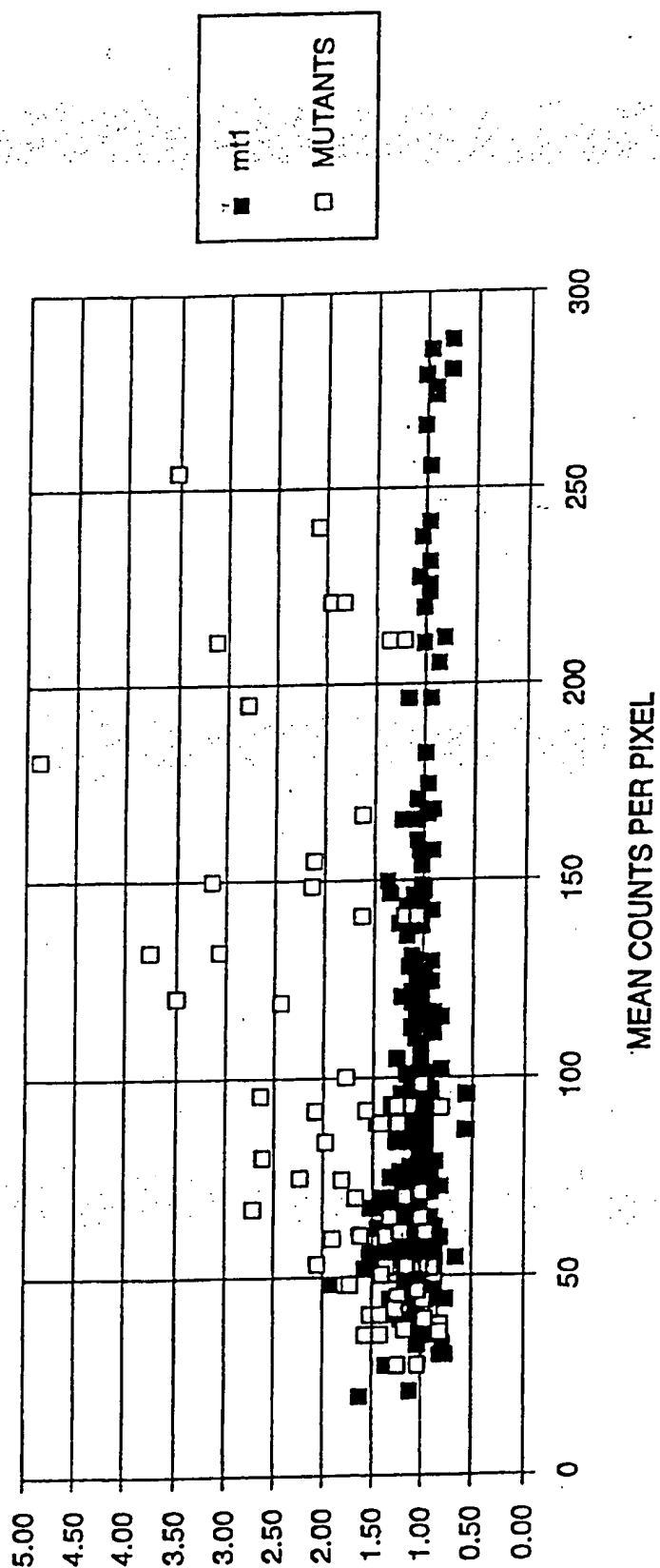


Fig. 15

Fig. 16

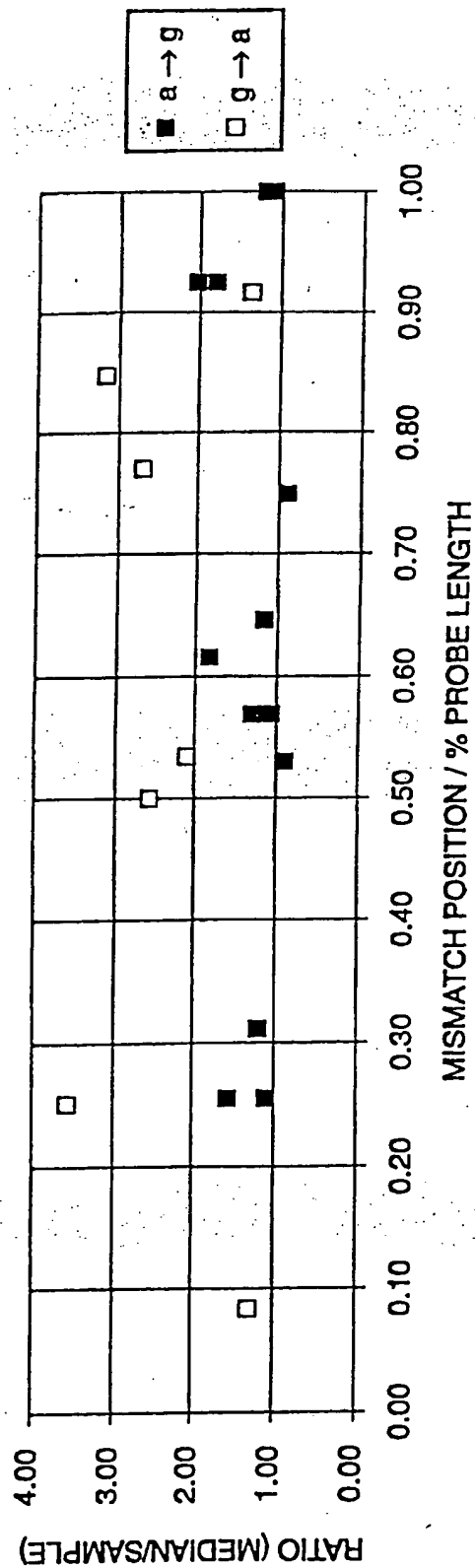
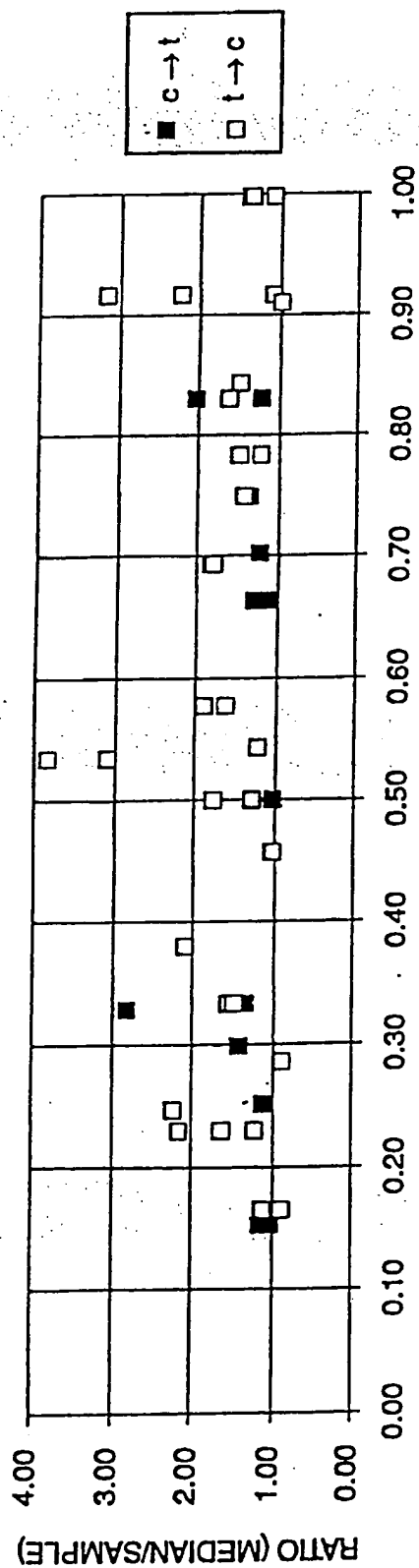


Fig. 17

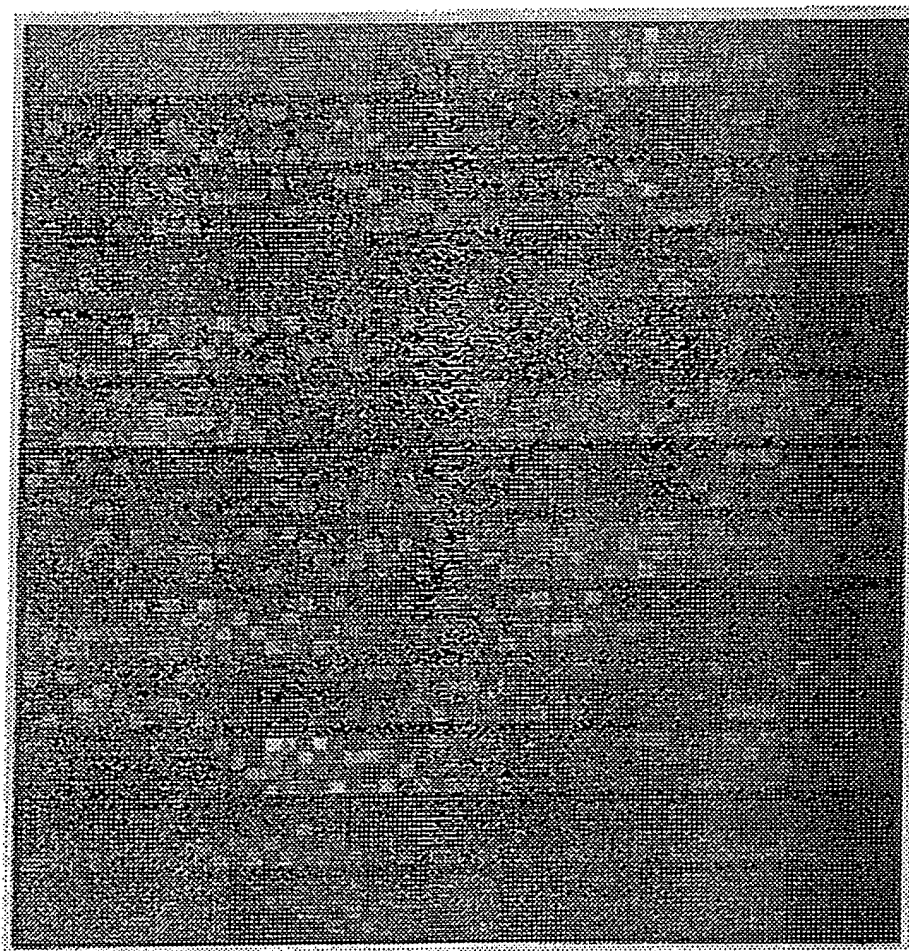


FIG. 18



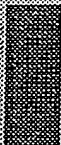

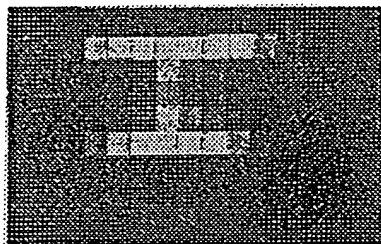
Position:	16319	152	263	344	
Change:	T->C	T->C	A->G	T->C	
Result:					T G C A

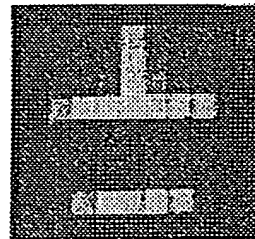
FIG. 19

Fig. 21

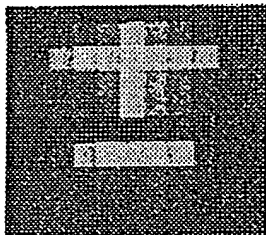
WT ("G" Substitution)
Target 12-mer



"A" Substitution 12-mer Target



"T" Substitution Target 12-mer



"C" Substitution Target 12-mer

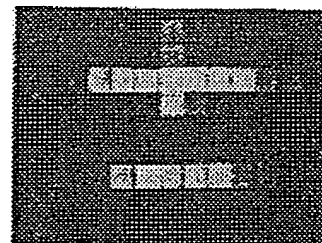
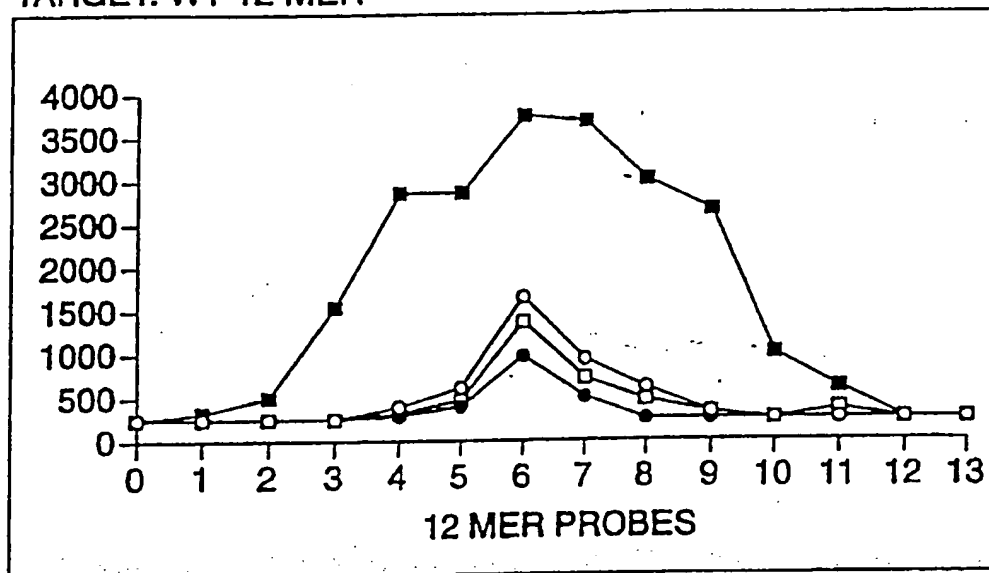


FIG. 22

Fig. 23A

TARGET: WT 12 MER



TARGET: T SUBSTITUTION IN POSITION 7

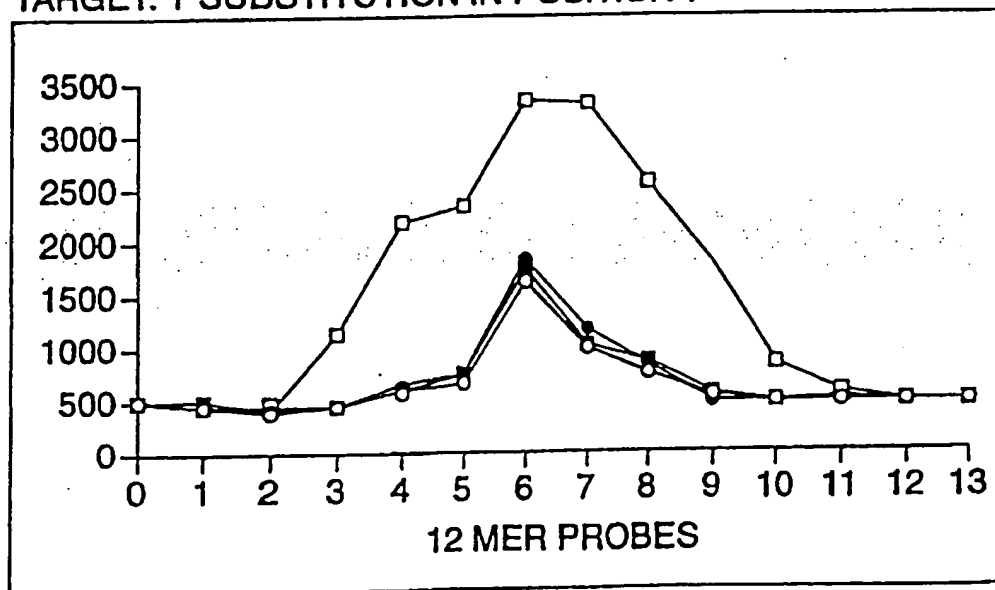
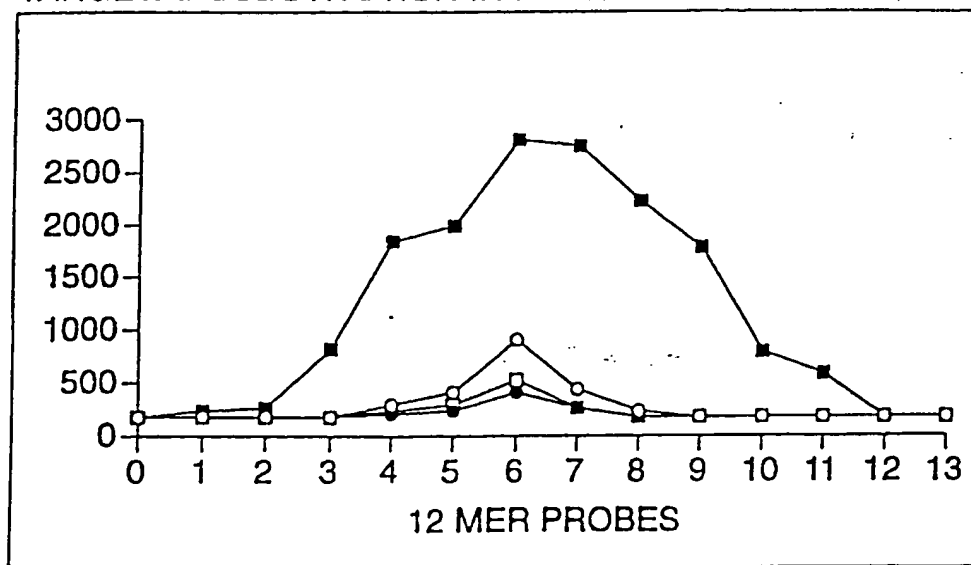
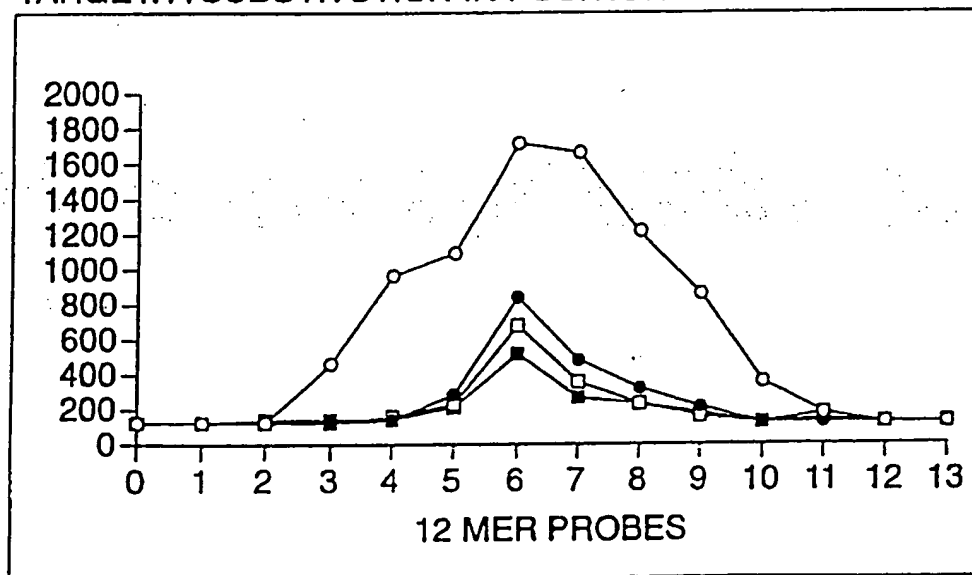


Fig. 23B

TARGET: C SUBSTITUTION IN POSITION 7



TARGET: A SUBSTITUTION IN POSITION 7



4:1 Mixture of WT and
"A" Substitution
12-mer Targets

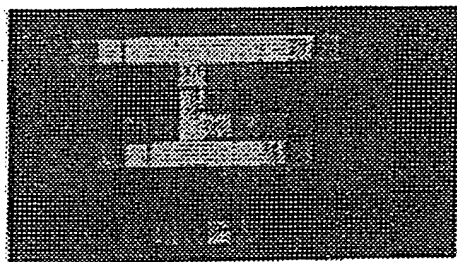
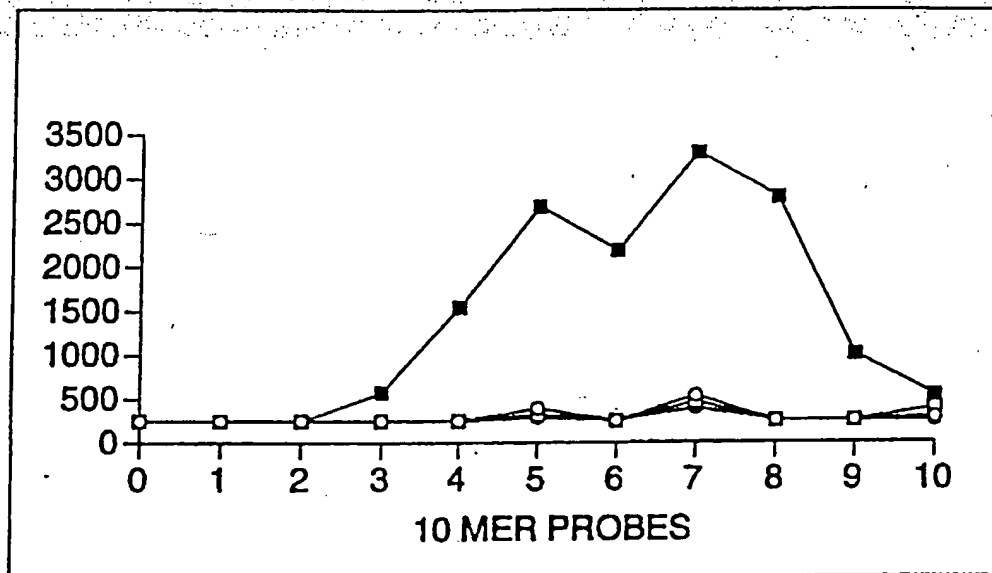


FIG. 24

Fig. 25A

TARGET: WT 12 MER



TARGET: T SUBSTITUTION IN POSITION 7

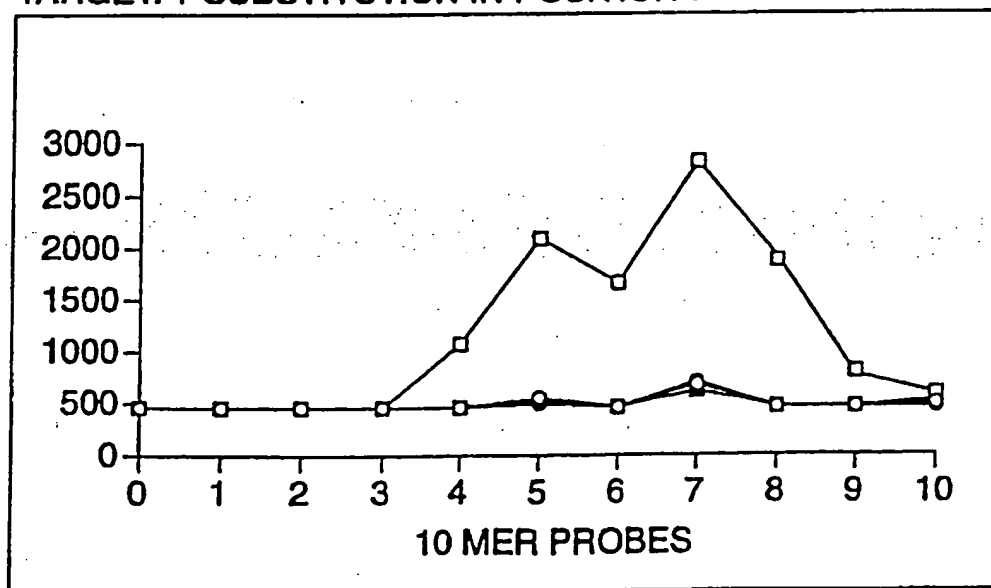
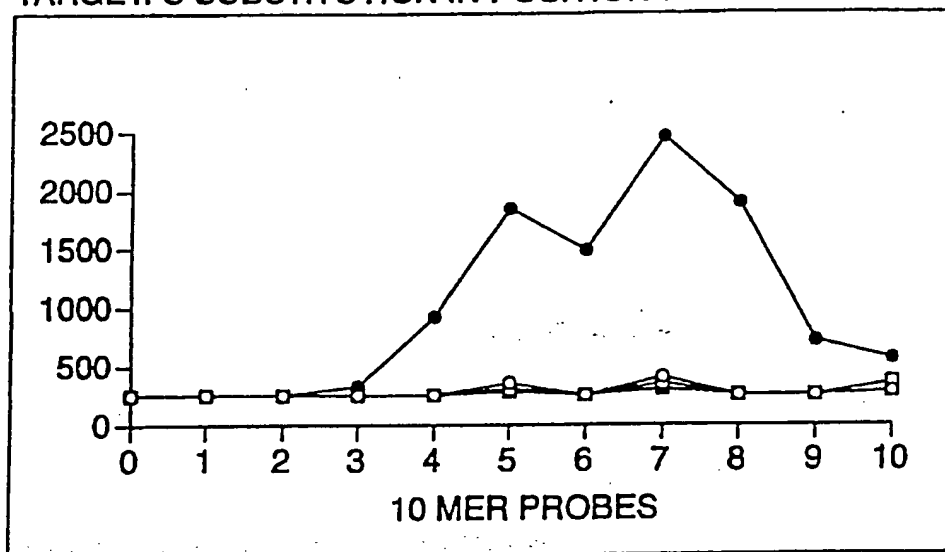
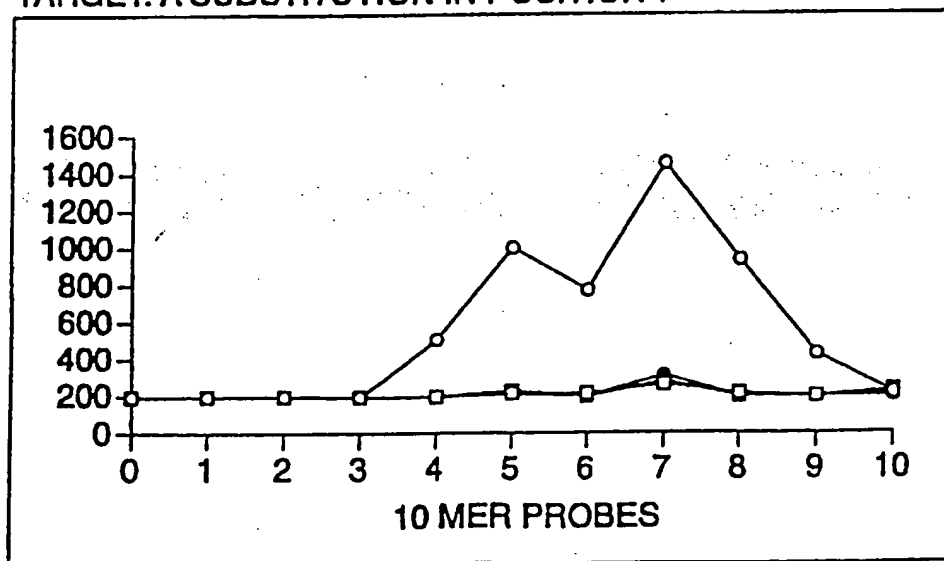


Fig. 25B

TARGET: C SUBSTITUTION IN POSITION 7



TARGET: A SUBSTITUTION IN POSITION 7



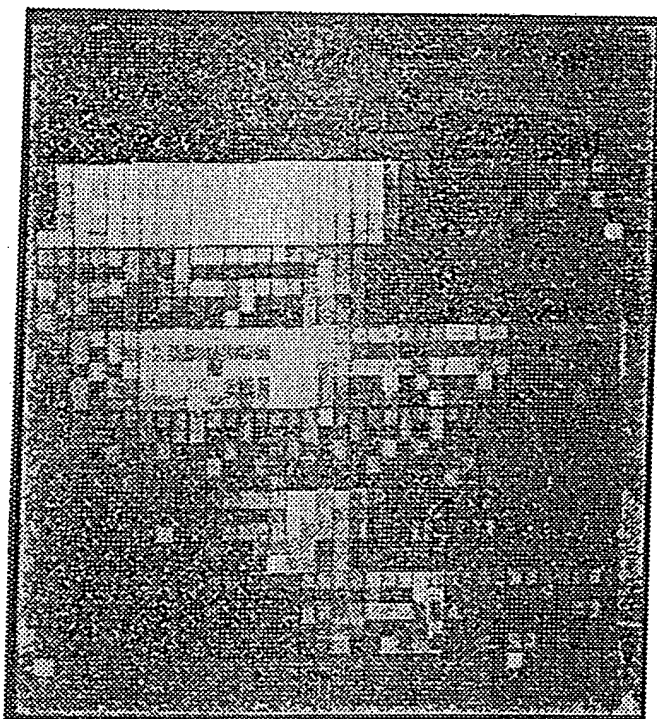


FIG. 26

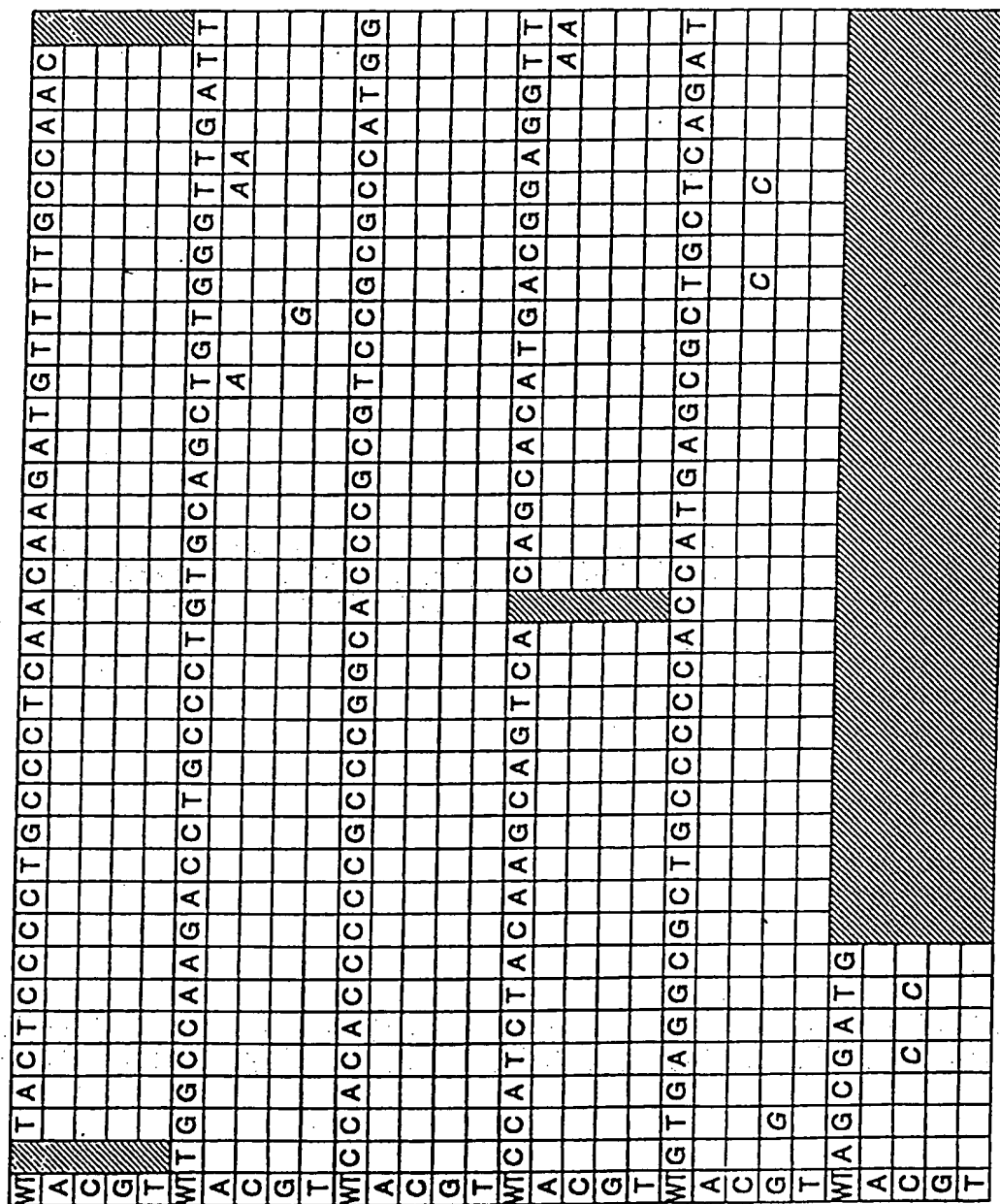


Fig. 27

Fig. 28

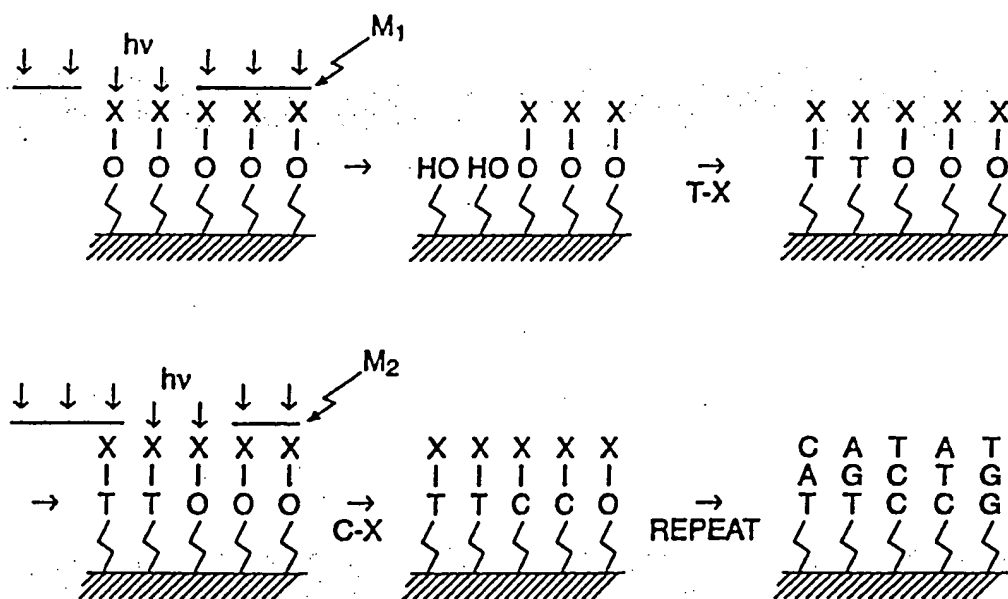
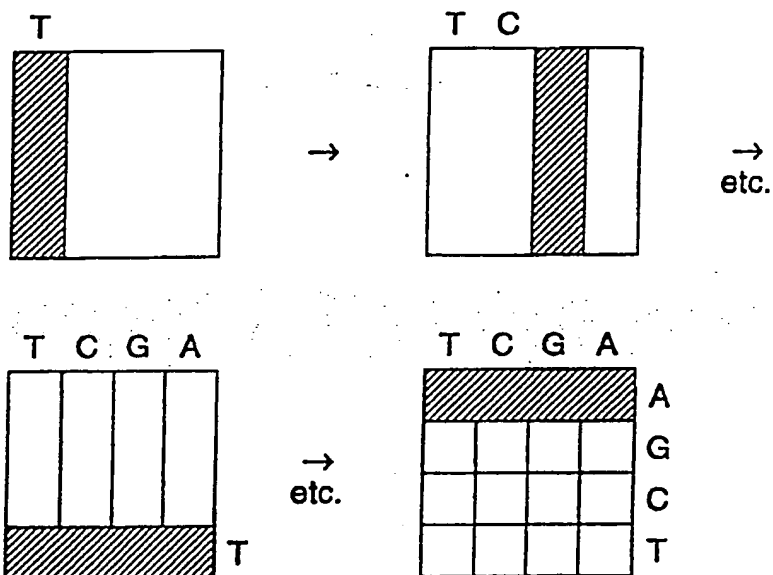


Fig. 29

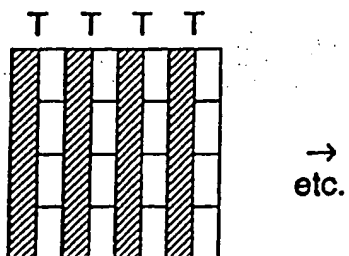
DIMERS:

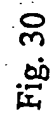


IN POLYNOMIAL NOTATION:

$$(T + C + A + G)^2 = \text{ALL DIMERS}$$

TRIMERS:





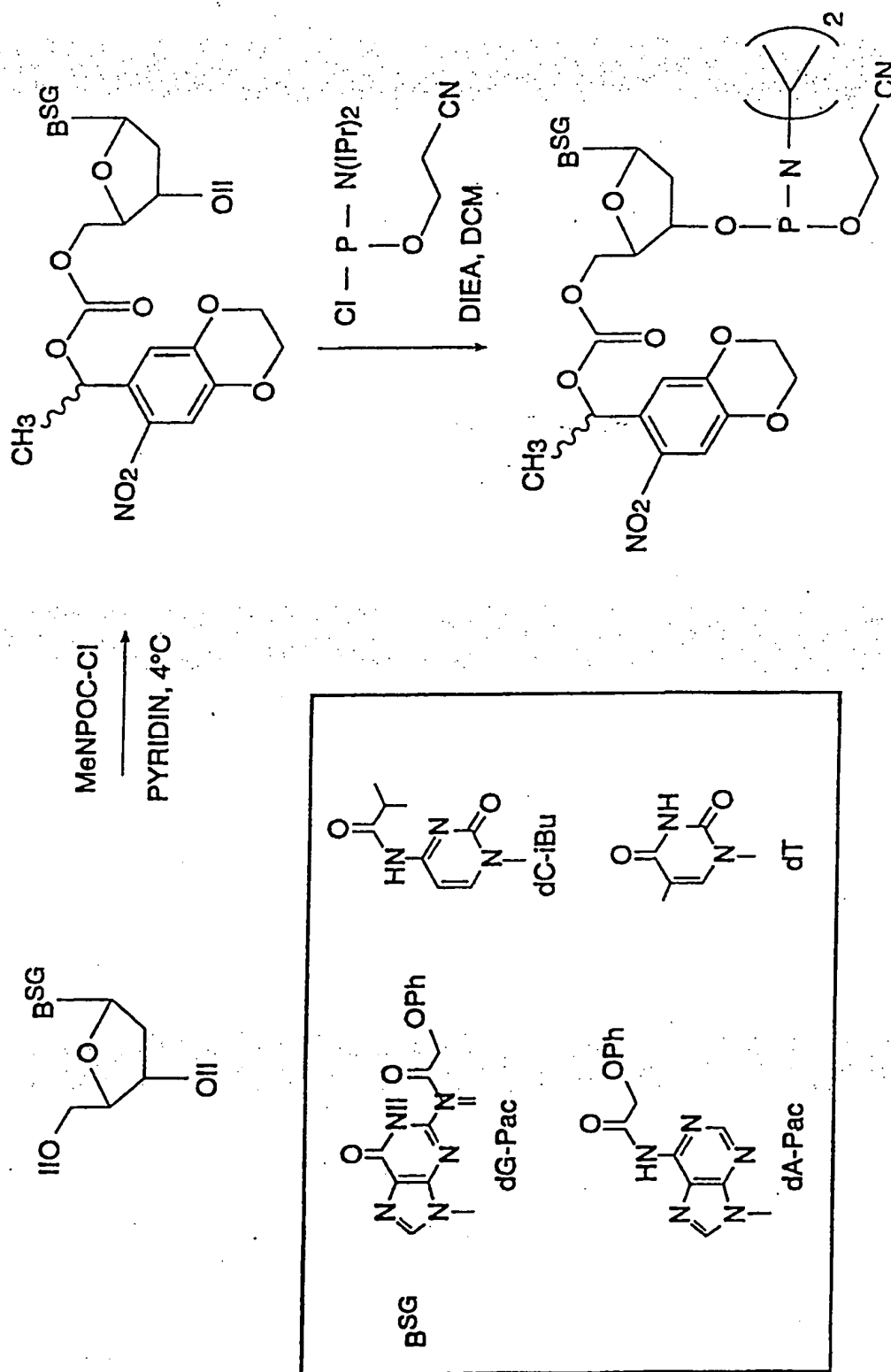


Fig. 31

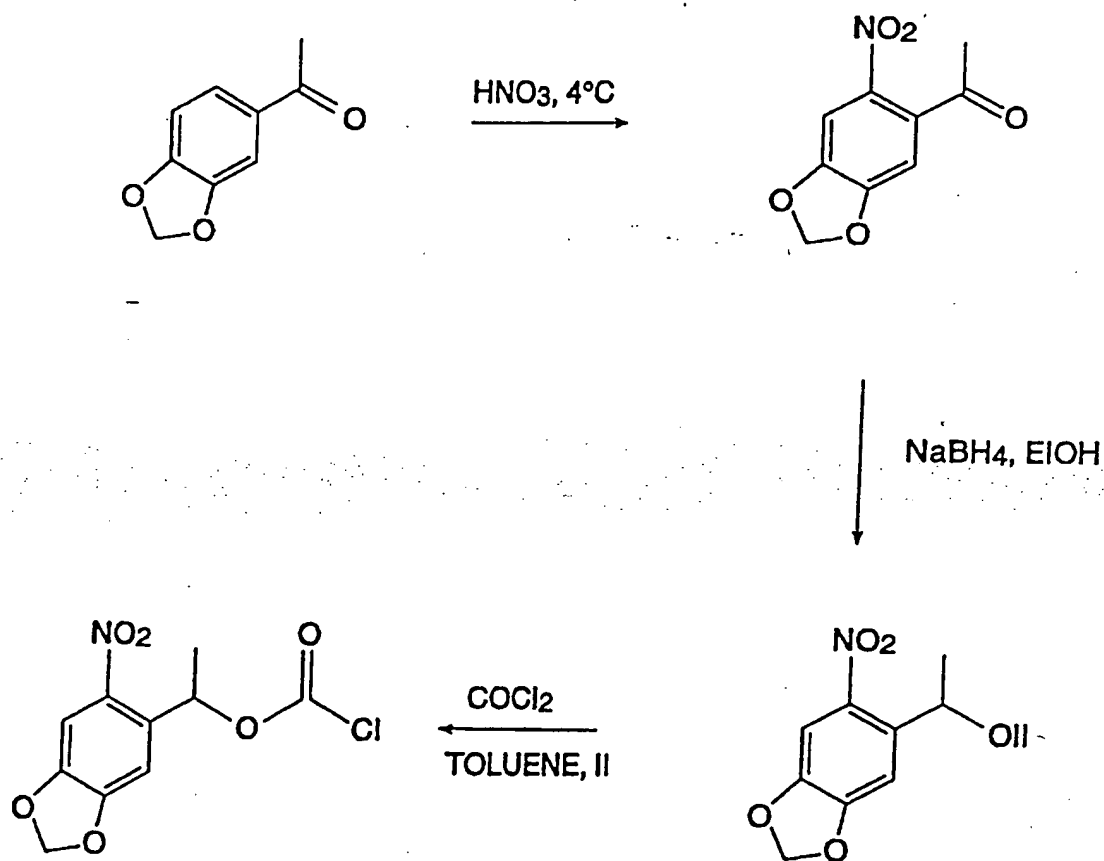


Fig. 32

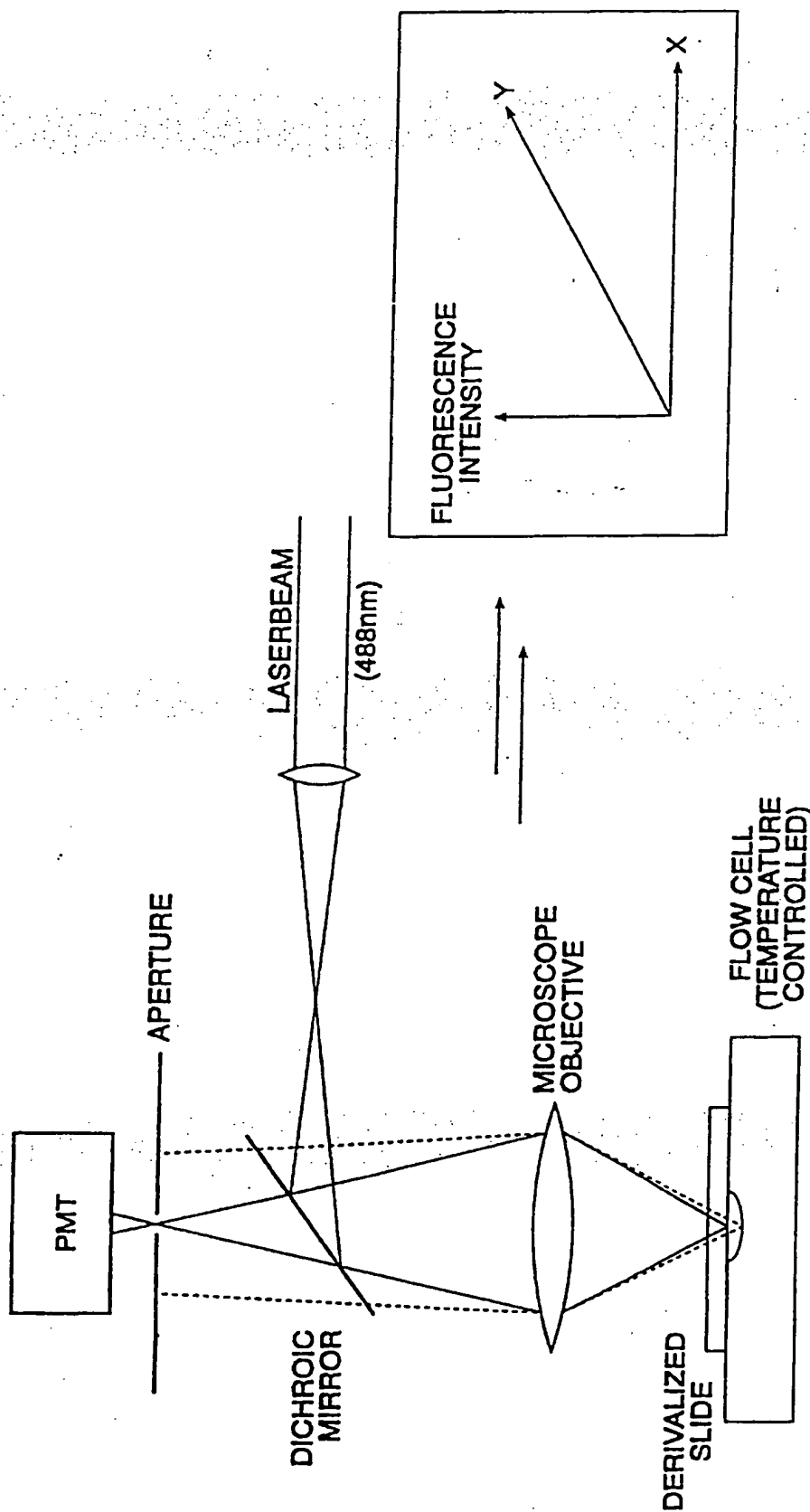


Fig. 33

ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS

CROSS-REFERENCE TO RELATED APPLICATION

This is a Continuation of application Ser. No. 08/143,312, filed Oct. 26, 1993, now abandoned, which is a continuation in part of U.S. patent application Ser. No. 082,937, filed 25 Jun. 1993, now abandoned, incorporated herein by reference.

Research leading to the invention was funded in part by NIH grant No. 1R01HG00813-01 and DOE grant No. DE-FG03-92-ER81275, and the government may have certain rights to the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention provides arrays of oligonucleotide probes immobilized in microfabricated patterns on silica chips for analyzing molecular interactions of biological interest. The invention therefore relates to diverse fields impacted by the nature of molecular interaction, including chemistry, biology, medicine, and medical diagnostics.

2. Description of Related Art

Oligonucleotide probes have long been used to detect complementary nucleic acid sequences in a nucleic acid of interest (the "target" nucleic acid). In some assay formats, the oligonucleotide probe is tethered, i.e., by covalent attachment, to a solid support, and arrays of oligonucleotide probes immobilized on solid supports have been used to detect specific nucleic acid sequences in a target nucleic acid. See, e.g., PCT patent publication Nos. WO 89/10977 and 89/11548. Others have proposed the use of large numbers of oligonucleotide probes to provide the complete nucleic acid sequence of a target nucleic but failed to provide an enabling method for using arrays of immobilized probes for this purpose. See U.S. Pat. Nos. 5,202,231 and 5,002,867 and PCT patent publication No. WO 93/17126.

The development of VLSIPS™ technology has provided methods for making very large arrays of oligonucleotide probes in very small arrays. See U.S. Pat. No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092, each of which is incorporated herein by reference. U.S. patent application Ser. No. 082,937, filed Jun. 25, 1993, describes methods for making arrays of oligonucleotide probes that can be used to provide the complete sequence of a target nucleic acid and to detect the presence of a nucleic acid containing a specific nucleotide sequence.

Microfabricated arrays of large numbers of oligonucleotide probes, called "DNA chips" offer great promise for a wide variety of applications. New methods and reagents are required to realize this promise, and the present invention helps meet that need.

SUMMARY OF THE INVENTION

The present invention provides methods for making high-density arrays of oligonucleotide probes on silica chips and for using those probe arrays to detect specific nucleic acid sequences contained in a target nucleic acid in a sample. The invention also provides arrays of oligonucleotide probes on DNA chips, in which the probes have specific sequences and locations in the array to facilitate identification of a specific target nucleic acid. In another aspect, the invention provides methods for detecting whether one or more specific sequences of a target nucleic acid in a sample varies from a

previously characterized sequence or reference sequence. The methods of the invention can be used to detect variations between a target and reference sequence, including single or multiple base substitutions, and deletions and insertions of bases, as well as detecting the presence, location, and sequence of other more complex variations between a target and reference sequence in a nucleic acid.

The present invention provides arrays of oligonucleotide probes immobilized on a solid support. The arrays are preferably synthesized directly on the support using VLSIPS™ technology, but other synthesis methods and immobilization of pre-synthesized oligonucleotide probes can be used to make the oligonucleotide probe arrays, called "DNA chips", of the invention. In general, these arrays comprise a set of oligonucleotide probes such that, for each base in a specific reference sequence, the set includes a probe (called the "wild-type" or "WT" probe) that is exactly complementary to a section of the reference sequence including the base of interest and four additional probes (called "substitution probes"), which are identical to the WT probe except that the base of interest has been replaced by one of a predetermined set (typically 4) of nucleotides. In the preferred embodiment, one of the four substitution probes is identical to the wild type probe; the other three are complementary to targets that have a single-base substitution at this position.

In another aspect, the invention relates to the arrangement of individual probes in the array. In one embodiment, the probes are arranged on the chip so that probes for a given position in the sequence are adjacent, and probes for adjacent positions in the reference sequence are also adjacent to one another on the chip. One method arranges the probes for a single base in a short column (alternately row) and arranges the columns in the order of the base position to form horizontal (alternately vertical) stripes. The wild-type and each of the substitution probes have specified positions within the column so that all the probes corresponding to an A substitution, for example, are in a single row. The stripes may be separated on the chip by a blank row or column.

The DNA chips of the invention can be made in a wide number of variations. For some applications, leaving out the wild-type row, leaving out unimportant bases, pooling bases, including insertion and deletion probes, varying the length of the probes within a set to make the probes have the same or similar Tm relative to the target or to avoid secondary structure, varying the mutation position, using multiple probes for a single mutation, providing replicate probes or arrays, placing blank "streets" (no probe) between rows, columns, or individual probes, and using control probes may be appropriate.

The present invention also provides DNA chips for detecting mutations associated with cystic fibrosis, including mutations in exons 4, 7, 9, 10, 11, 20, and 21 of the CFTR gene. The invention also provides DNA chips for detecting mutations in the p53 gene, a gene in which mutations are known to be associated with a wide variety of cancers. Other DNA chips of the invention provide probe arrays for detecting specific sequences of mitochondrial DNA, useful for identification and forensic purposes. The invention also provides DNA chips for detecting specific sequences of nucleotides or mutations associated with the acquisition of a drug resistant phenotype in an infectious organism, such as rifampicin or other drug resistant TB strains and HIV, in which mutations in an RNA polymerase gene are known to give rise to drug resistance.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows how the tiling method of the invention defines a set of DNA probes relative to a target nucleic acid.

In the figure, the target is a DNA molecule, the probes are single-stranded nucleic acids 16 nucleotides in length, and only a portion of the probes defined by the method is shown.

FIG. 2 shows an illustrative tiled array of the invention with probes for the detection of point mutations. The base at the position of substitution in each of the wild-type probes is shown in the wild-type lane, and the shading shows the location of the substitution probe having the wild-type sequence. The SEQ ID. NOS. corresponding to the two peptide sequences shown in the top portion of FIG. 2 are 311 and 312, respectively. The SEQ ID. NOS. corresponding to the five peptide sequences listed at the bottom of FIG. 2 are 313, 314, 315, 313, and 316, respectively.

FIG. 3, in panels A, B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to a wild-type target; in panel C, the chip was hybridized to a mutant $\Delta F508$ target; and in panel B, the chip was hybridized to a mixture of the wild-type and mutant targets. The SEQ ID. NOS. corresponding to the four peptide sequences shown in FIG. 3 are 317-320, respectively.

FIG. 4, in sheets 1-3, corresponding to panels A, B, and C of FIG. 3, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest"). The SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 1 of FIG. 4 are 321 and 318, respectively; the SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 2 of FIG. 4 are 322 and 318, respectively; and the SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 3 of FIG. 4 are 323 and 318, respectively.

FIG. 5, in panels A, B, and C, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to the wt480 target; in panel C, the chip was hybridized to the mu480 target; and in panel B, the chip was hybridized to a mixture of the wild-type and mutant targets. The SEQ ID. NOS. corresponding to the peptide sequences shown in FIG. 5 are 324-327, respectively.

FIG. 6, in sheets 1-3, corresponding to panels A, B, and C of FIG. 5, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest"). The SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 1 of FIG. 6 are 328 and 329, respectively; the SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 2 of FIG. 6 are 330 and 329, respectively; and the SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 3 of FIG. 6 are 331 and 329, respectively.

FIG. 7, in panels A and B, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to nucleic acid derived

from the genomic DNA of an individual with wild-type $\Delta F508$ sequences; in panel B, the target nucleic acid originated from a heterozygous (with respect to the $\Delta F508$ mutation) individual.

FIG. 8, in sheets 1 and 2, corresponding to panels A and B of FIG. 7, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest"). The SEQ ID. NOS. corresponding to the two peptide sequences shown in sheet 2 of FIG. 8 are 332 and 318, respectively.

FIG. 9 shows the human mitochondrial genome; "O_H" is the H strand origin of replication, and arrows indicate the cloned unshaded sequence.

FIG. 10 shows the image observed from application of a sample of mitochondrial DNA derived nucleic acid (from the mt4 sample) on a DNA chip.

FIG. 11 is similar to FIG. 10 but shows the image observed from the mt5 sample.

FIG. 12 shows the predicted difference image between the mt4 and mt5 samples on the DNA chip based on mismatches between the two samples and the reference sequence.

FIG. 13 shows the actual difference image observed for the mt4 and mt5 samples.

FIG. 14, in sheets 1 and 2, shows a plot of normalized intensities across rows 10 and 11 of the array and a tabulation of the mutations detected.

FIG. 15 shows the discrimination between wild-type and mutant hybrids obtained with the chip. A median of the six normalized hybridization scores for each probe was taken; the graph plots the ratio of the median score to the normalized hybridization score versus mean counts. A ratio of 1.6 and mean counts above 50 yield no false positives.

FIG. 16 illustrates how the identity of the base mismatch may influence the ability to discriminate mutant and wild-type sequences more than the position of the mismatch within an oligonucleotide probe. The mismatch position is expressed as % of probe length from the 3'-end. The base change is indicated on the graph.

FIG. 17 provides a 5' to 3' sequence listing of one target corresponding to the probes on the chip. X is a control probe. Positions that differ in the target (i.e., are mismatched with the probe at the designated site) are in bold. The SEQ ID. NO. corresponding to the peptide sequence shown in FIG. 17 is 333.

FIG. 18 shows the fluorescence image produced by scanning the chip described in FIG. 17 when hybridized to a sample.

FIG. 19 illustrates the detection of 4 transitions in the target sequence relative to the wild-type probes on the chip in FIG. 18.

FIG. 20 shows the alignment of some of the probes on a p³³ DNA chip with a 12-mer model target nucleic acid. The SEQ ID. NOS. corresponding to the fourteen peptide sequences shown in FIG. 20 are 334-347, respectively.

FIG. 21 shows a set of 10-mer probes for a p53 exon 6 DNA chip. The SEQ ID. NOS. corresponding to the thirteen peptide sequences shown in FIG. 21 are 334 and 348-359, respectively.

FIG. 22 shows that very distinct patterns are observed after hybridization of p53 DNA chips with targets having different 1 base substitutions. In the first image in FIG. 22, the 12-mer probes that form perfect matches with the wild-type target are in the first row (top). The 12-mer probes with single base mismatches are located in the second, third, and fourth rows and have much lower signals.

FIG. 23, in graphs 2, 3, and 4, graphically depicts the data in FIG. 22. On each graph, the X ordinate is the position of the probe in its row on the chip, and the Y ordinate is the signal at that probe site after hybridization.

FIG. 24 shows the results of hybridizing mixed target populations of WT and mutant p53 genes to the p53 DNA chip.

FIG. 25, in graphs 1-4, shows (see FIG. 23 as well) the hybridization efficiency of a 10-mer probe array as compared to a 12-mer probe array.

FIG. 26 shows an image of a p53 DNA chip hybridized to a target DNA.

FIG. 27 illustrates how the actual sequence was read from the chip shown in FIG. 26. Gaps in the sequence of letters in the WT rows correspond to control probes or sites. Positions at which bases are miscalled are represented by letters in italic type in cells corresponding to probes in which the WT bases have been substituted by other bases. The SEQ ID. NO. corresponding to the peptide sequence shown in FIG. 27 is 360.

FIG. 28 illustrates the VLSIPS™ technology as applied to the light directed synthesis of oligonucleotides. Light (hv) is shone through a mask (M₁) to activate functional groups (—OH) on a surface by removal of a protecting group (X). Nucleoside building blocks protected with photoremovable protecting groups (T-X, C-X) are coupled to the activated areas. By repeating the irradiation and coupling steps, very complex arrays of oligonucleotides can be prepared.

FIG. 29 illustrates how the VLSIPS™ process can be used to prepare "nucleoside combinatorials" or oligonucleotides synthesized by coupling all four nucleosides to form dimers, trimers, etc.

FIG. 30 shows the deprotection, coupling, and oxidation steps of a solid phase DNA synthesis method.

FIG. 31 shows an illustrative synthesis route for the nucleoside building blocks used in the VLSIPS™ method.

FIG. 32 shows a preferred photoremovable protecting group, MeNPOC, and how to prepare the group in active form.

FIG. 33 illustrates an illustrative detection system for scanning a DNA chip.

DETAILED DESCRIPTION OF THE INVENTION

Using the VLSIPS™ method, one can synthesize arrays of many thousands of oligonucleotide probes on a substrate, such as a glass slide or chip. The method can be used, for instance, to synthesize "combinatorial" arrays consisting of, for example, all possible octanucleotides. Such arrays can be used for primary sequencing-by-hybridization on genomic DNA fragments or other nucleic acids or to detect mutations in a target nucleic acid for which the normal or "wild-type" nucleotide sequence is already known. Using the preferred method of the invention, one employs a strategy called "tiling" to synthesize specific sets of probes or at spatially-defined locations on a substrate, creating the novel probe arrays and "DNA chips" of the invention.

To illustrate the tiling method of the invention, consider the problem of detecting mutations at one or more position

in the nucleotide sequence of a target nucleic acid with oligonucleotide probes of defined length. The length (L) of the probe is typically expressed as the number of nucleotides or bases in a single-stranded nucleic acid probe. For purposes of the present invention, lengths ranging from 12 to 18 bases are preferred, although shorter and longer lengths can also be employed. To employ the tiling method, one synthesizes a set of probes defined by the particular nucleotide sequence of interest in the target nucleic acid. For each base in the target DNA segment, one synthesizes a probe complementary to the subsequence of the target nucleic acid beginning at that base and ending L-1 bases to the 3'-side (see FIG. 1).

In a preferred embodiment of the invention, the probes are arranged (either by immobilization, typically by covalent attachment, of a pre-synthesized probe or by synthesis of the probe on the substrate) on the substrate or chips in lanes stretching across the chip and separated, and these lanes are in turned arranged in blocks of preferably 5 lanes, although blocks of other sizes will have useful application, as will be apparent from the following illustration. The first of these five lanes, called the "wild-type lane", contains probes arranged in order of sequence, and all of the probes are complementary to a specified wild-type nucleic acid sequence. The other four lanes contain probe sets for detecting all possible single-base mutations in the defined sequence; in turn, these probe sets are defined by a position of potential non-complementarity in the probe relative to the target (i.e., a single base mismatch) and the identity of the nucleotide in the probe at that position (i.e., whether the nucleotide is an A, C, G, or T nucleotide). The position of mismatch, also called the position of substitution, is preferably selected to be near the center of the probes, i.e., position 7 of a probe of L=15.

For each probe in the wild-type lane, one synthesizes four probes (one for each of the lanes other than the wild-type lane). Three of these four probes is identical to the corresponding wild-type probe but for the base at the position of substitution, and the remaining probe is identical to the wild-type probe. This set of four substitution probes is preferably placed in a column directly below (or above) the corresponding wild-type probe, thus creating an A-lane, a C-lane, a G-lane, and a T-lane. FIG. 2 shows an illustrative tiled array of the invention with probes for the detection of point mutations. The base at the position of substitution in each of the wild-type probes is shown in the wild-type lane, and the shading shows the location of the substitution probe having the wild-type sequence. Below are the probes that would be placed in the column marked by the arrow if the probe length were 15 and the position of substitution were 7.

3'-CCGACTGCAGTCGTT (SEQ. ID. NO:1)

3'-CCGACTACAGTCGTT (SEQ. ID. NO:2)

3'-CCGACTCCAGTCGTT (SEQ. ID. NO:3)

3'-CCGACTGCAGTCGTT (SEQ. ID. NO:1)

3'-CCGACTTCAGTCGTT (SEQ. ID. NO:4)

Thus, the substitution lanes occupy four of the five lanes separating successive wild-type lanes on the chip; the blocks of five lanes can be separated by a sixth lane for measurement of background signals.

The DNA chips of the invention have a wide variety of applications. In one embodiment, the DNA chip is used to select an optimal probe from an array of probes. In this embodiment, an array of probes of variable length and sequences is synthesized and then hybridized to a target nucleic acid of known sequence. The pattern of hybridization reveals the optimal length and sequence composition of

probes to detect a particular mutation or other specific sequence of nucleotides. In some circumstances, i.e., target nucleic acids with repeated sequences or with high G/C content, very long probes may be required for optimal detection. In one embodiment for detecting specific sequences in a target nucleic acid with a DNA chip, repeat sequences are detected as follows. The chip comprises probes of length sufficient to extend into the repeat region varying distances from each end. The sample, prior to hybridization, is treated with a labeled oligonucleotide that is complementary to a repeat region but shorter than the full length of the repeat. The target nucleic acid is labeled with a second, distinct label. After hybridization, the chip is scanned for probes that have bound both the labeled target and the labeled oligonucleotide probe; the presence of such bound probes shows that at least two repeat sequences are present.

A variety of methods can be used to enhance detection of labeled targets bound to a probe on the array. In one embodiment, the protein MutS (from *E. coli*) or equivalent proteins such as yeast MSH1, MSH2, and MSH3; mouse Rep-3, and *Streptococcus* Hex-A, is used in conjunction with target hybridization to detect probe-target complex that contain mismatched base pairs. The protein, labeled directly or indirectly, can be added to the chip during or after hybridization of target nucleic acid, and differentially binds to homo- and heteroduplex nucleic acid. A wide variety of dyes and other labels can be used for similar purposes. For instance, the dye YOYO-1 is known to bind preferentially to nucleic acids containing sequences comprising runs of 3 or more G residues.

The DNA chips produced by the methods of the invention can be used to study and detect mutations in exons of human genes of clinical interest, including point mutations and deletions. In the following sections, the method of the invention is illustrated by the detection of mutations in a variety of clinically and medically significant human nucleic acid sequences. Thus, the invention is illustrated first with respect to the preparation of DNA chips for the detection of mutations associated with cystic fibrosis, then with DNA chips for the detection of human mitochondrial DNA sequences, then with DNA chips for the detection of mutations in the human p53 gene associated with cancer, and finally with respect to the detection of mutations in the HIV RT gene associated with drug resistance.

Detection of Cystic Fibrosis Mutations with DNA Chips

A number of years ago, cystic fibrosis, the most common severe autosomal recessive disorder in humans, was shown to be associated with mutations in a gene thereafter named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The sequences of the exons and parts of the introns in the gene are known, as are the changes corresponding to several hundred known mutations. Several tests have been developed for detecting the most frequent of these mutations. The present invention provides CFTR gene oligonucleotide arrays (DNA chips) that can be used to identify mutations in the CFTR gene rapidly and efficiently.

The methods used to make the high-density DNA chips of the invention allow probes for long stretches of DNA coding regions to be directly "written" onto the chips in the form of sets of overlapping oligonucleotides. These methods have been used to develop a number of useful CFTR gene chips, one illustrative chip bears an array of 1296 probes covering the full length of exon 10 of the CFTR gene arranged in a 36x36 array of 356 μm elements. The probes in the array can have any length, preferably in the range of from 10 to 18 residues and can be used to detect and sequence any single-

base substitution and any deletion within the 192-base exon, including the three-base deletion known as ΔF508 . As described in detail below, hybridization of sub-nanomolar concentrations of wild-type and ΔF508 oligonucleotide target nucleic acids labeled with fluorescein to these arrays produces highly specific signals (detected with confocal scanning fluorescence microscopy) that permit discrimination between mutant and wild-type target sequences in both homozygous and heterozygous cases. The method and chips of the invention can also be used to detect other known mutations in the CFTR gene, as described in detail below.

The most common cystic fibrosis mutation is known as ΔF508 , because the mutation is a three-base deletion that results in the removal of amino acid #508 from the CFTR protein. The present invention provides DNA chips for detecting ΔF508 , one such chip results from applying the tiling method to exon 10 of the CFTR gene, the exon to which ΔF508 has been mapped. The tiling method involved the synthesis of a set of probes of a selected length in the range of from 10 to 18 bases and complementary to subsequences of the known wild-type CFTR sequence starting at a position a few bases into the intron on the 5'-side of exon 10 and ending a few bases into the intron on the 3'-side. There was a probe for each possible subsequence of the given segment of the gene, and the probes were organized into a "lane" in such a way that traversing the lane from the upper left-hand corner of the chip to the lower righthand corner corresponded to traversing the gene segment base-by-base from the 5'-end. The lane containing that set of probes is, as noted above, called the "wild-type lane."

Relative to the wild-type lane, a "substitution" lane, called the "A-lane", was synthesized on the chip. The A-lane probes were identical in sequence to an adjacent (immediately below the corresponding) wild-type probe but contained, regardless of the sequence of the wild-type probe, a dA residue at position 7 (counting from the 3'-end). In similar fashion, substitution lanes with replacement bases dC, dG, and dT were placed onto the chip in a "C-lane," a "G-lane," and a "T-lane," respectively. A sixth lane on the chip consisted of probes identical to those in the wild-type lane but for the deletion of the base in position 7 and restoration of the original probe length by addition to the 5'-end the base complementary to the gene at that position.

The four substitution lanes enable one to deduce the sequence of a target exon 10 nucleic acid from the relative intensities with which the target hybridizes to the probes in the various lanes. The probe organization on the chip can be conveniently columnar, and the set of probes consisting of a wild-type probe and four corresponding substitution probes is referred to as a "column set." One and only one of the four substitution probes in a column set has exactly the same sequence as the wild-type probe in the set. Those of skill in the art will appreciate that, in other embodiments of the invention, one could delete one or more lanes or columns and still benefit from the invention. Various versions of such exon 10 DNA chips were made as described above with probes 15 bases long, as well as chips with probes 10, 14, and 18 bases long. For the results described below, the probes were 15 bases long, and the position of substitution was 7 from the 3'-end.

To demonstrate the ability of the chip to distinguish the ΔF508 mutation from the wild-type, two synthetic target nucleic acids were made. The first, a 39-mer complementary to a subsequence of exon 10 of the CFTR gene having the three bases involved in the ΔF508 mutation near its center, is called the "wild-type" or wt508 target, corresponds to positions 111-149 of the exon, and has the sequence shown below:

5'-CATTAAAGAAAATATCATCTTTGGTGTTCCTATGATGA (SEQ. ID NO: 5).

The second, a 36-mer probe derived from the wild-type target by removing those same three bases, is called the "mutant" target or mu508 target and has the sequence shown below, first with dashes to indicate the deleted bases, and then without dashes but with one base underlined (to indicate the base detected by the T-lane probe, as discussed below):

5' - C A T T A A A G A A A A T A T C A T - - - 10
TGGTGTTCCTATGATGA; (SEQ. ID NO:6)
5'-CATTAAAGAAAATATCATTGGTGTTCCTATGATGA.
(SEQ. ID NO:7)

Both targets were labeled with fluorescein at the 5'-end.

In three separate experiments, the wild-type target, the mutant target, and an equimolar mixture of both targets was exposed (0.1 nM wt508, 0.1 nM mu508, and 0.1 nM wt508 plus 0.1 nM mu508, respectively, in a solution compatible with nucleic acid hybridization) to a CF chip. The hybridization mixture was incubated overnight at room temperature, and then the chip was scanned on a reader (a confocal fluorescence microscope in photon-counting mode; images of the chip were constructed from the photon counts) at several successively higher temperatures while still in contact with the target solution. After each temperature change, the chip was allowed to equilibrate for approximately one-half hour before being scanned. After each set of scans, the chip was exposed to denaturing solvent and conditions to wash, i.e., remove target that had bound, the chip so that the next experiment could be done with a clean chip.

The results of the experiments are shown in FIGS. 3, 4, 5, and 6. FIG. 3, in panels A, B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to a wild-type target; in panel C, the chip was hybridized to a mutant delta 508 target; and in panel B, the chip was hybridized to a mixture of the wild-type and mutant targets. FIG. 4, in sheets 1-3, corresponding to panels A, B, and C of FIG. 3, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest").

These figures show that, for the wild-type target and the equimolar mixture of targets, the substitution probe with a nucleotide sequence identical to the corresponding wild-type probe bound the most target, allowing for an unambiguous assignment of target sequence as shown by letters near the points on the curve. The target wt508 thus hybridized to the probes in the wild-type lane of the chip, although the strength of the hybridization varied from probe-to-probe, probably due to differences in melting temperature. The sequence of most of the target can thus be read directly from the chip, by inference from the pattern of hybridization in the lanes of substitution probes (if the target hybridizes most intensely to the probe in the A-lane, then one infers that the target has a T in the position of substitution, and so on).

For the mutant target, the sequence could similarly be called on the 3'-side of the deletion. However, the intensity of binding declined precipitously as the point of substitution approached the site of the deletion from the 3'-end of the target, so that the binding intensity on the wild-type probe

whose point of substitution corresponds to the T at the 3'-end of the deletion was very close to background. Following that pattern, the wild-type probe whose point of substitution corresponds to the middle base (also a T) of the deletion bound still less target. However, the probe in the T-lane of that column set bound the target very well.

Examination of the sequences of the two targets reveals that the deletion places an A at that position when the sequences are aligned at their 3'-ends and that the T-lane probe is complementary to the mutant target with but two mismatches near an end (shown below in lower-case letters, with the position of substitution underlined):

Target: 5'-CATTAAAGAAAATATCATTGGTGTTCCTATGATGA

Probe: 3'-TagTAGTAACCACAA (SEQ. ID NO:8)

Thus the T-lane probe in that column set calls the correct base from the mutant sequence. Note that, in the graph for the equimolar mixture of the two targets, that T-lane probe binds almost as much target as does the A-lane probe in the same column set, whereas in the other column sets, the probes that do not have wild-type sequence do not bind target at all as well. Thus, that one column set, and in particular the T-lane probe within that set, detects the Δ 508 mutation under conditions that simulate the homozygous case and also conditions that simulate the heterozygous case.

The present invention thus provides individual probes, sets of probes, and arrays of probe sets on chips, in specific patterns, as the probes provide important benefits for detecting the presence of specific exon 10 sequences. The sequences of several important probes of the invention are shown below. In each case, the letter "X" stands for the point of substitution in a given column set, so each of the sequences actually represents four probes, with A, C, G, and T, respectively, taking the place of the "X." Sets of shorter probes derived from the sets shown below by removing up to five bases from the 5'-end of each probe and sets of longer probes made from this set by adding up to three bases from the exon 10 sequence to the 5'-end of each probe, are also useful and provided by the invention.

3'-TTTATAXTAGAAACC (SEQ. ID NO:9)
3'-TTATAGXAGAAACCA (SEQ. ID NO:10)
3'-TATAGTXGAAACCAC (SEQ. ID NO:11)
3'-ATAGTAXAAACCACA (SEQ. ID NO:12)
3'-TAGTAGXAACCACAA (SEQ. ID NO:13)
3'-AGTAGAXACCACAAA (SEQ. ID NO:14)
3'-GTAGAAXCCACAAAG (SEQ. ID NO:15)
3'-TAGAAAXCACAAAGG (SEQ. ID NO:16)
3'-AGAAACXACAAAGGA (SEQ. ID NO:17)

Although in this example the sequence could not be reliably deduced near the ends of the target, where there is not enough overlap between target and probe to allow effective hybridization, and around the center of the target, where hybridization was weak for some other reason, perhaps high AT-content, the results show the method and the probes of the invention can be used to detect the mutation of interest. The mutant target gave a pattern of hybridization that was very similar to that of the wt508 target at the ends, where the two share a common sequence, and very different in the middle, where the deletion is located. As one scans the image from right to left, the intensity of hybridization of the target to the probes in the wild-type lane drops off much more rapidly near the center of the image for mu508 than for wt508; in addition, there is one probe in the T-lane that hybridizes intensely with mu508 and hardly at all with wt508. The results from the equimolar mixture of the two targets, which represents the case one would encounter in testing a heterozygous individual for the mutation, are a

blend of the results for the separate targets, showing the power of the invention to distinguish a wild-type target sequence from one containing the Δ F508 mutation and to detect a mixture of the two sequences.

The results above clearly demonstrate how the DNA chips of the invention can be used to detect a deletion mutation, Δ F508; another model system was used to show that the chips can also be used to detect a point mutation as well. One of the more frequent mutations in the CFTR gene is G480C, which involves the replacement of the G in position 46 of exon 10 by a T, resulting in the substitution of a cysteine for the glycine normally in position #480 of the CFTR protein. The model target sequences included the 21-mer probe wt480 to represent the wild-type sequence at positions 37-55 of exon 10: 5'-CCTTCAGAGGGTAAAATTAAG (SEQ. ID NO:18) and the 21-mer probe mu480 to represent the mutant sequence: 5'-CCTTCAGAGTGTAAAATTAAG (SEQ. ID NO:19).

terms. The wild-type sequence could easily be read from the chip, but the probe that bound the mu480 target so well when only the mu480 target was present also bound it well when both the mutant and wild-type targets were present in a mixture, making the hybridization pattern easily distinguishable from that of the wild-type target alone. These results again show the power of the DNA chips of the invention to detect point mutations in both homo- and heterozygous individuals.

To demonstrate clinical application of the DNA chips of the invention, the chips were used to study and detect mutations in nucleic acids from genomic samples. Genomic samples from a individual carrying only the wild-type gene and an individual heterozygous for Δ F508 were amplified by PCR using exon 10 primers containing the promoter for T7 RNA polymerase. Illustrative primers of the invention are shown below.

Exon	Name	Sequence	
10	CF19-T7	TAATACGACTCACTATAGGGAGatgacctaatatgatgggttt	(SEQ. ID. NO:20)
10	CF110c-T7	TAATACGACTCACTATAGGGAGatgtgtgagggttcataatgc	(SEQ. ID. NO:21)
10	CF110c-T3	CTCGGAATTAACCCCTCACTAAAGGtagtgaagggttcataatg	(SEQ. ID. NO:22)
10, 11	CF110-T7	TAATACGACTCACTATAGGGAGagcatactaaagtgccttc	(SEQ. ID. NO:23)
11	CF111c-T7	TAATACGACTCACTATAGGGAGacatgaatgacattacagcaa	(SEQ. ID. NO:24)
11	CF111c-T3	CGGAATTAACCCCTCACTAAAGGacatgaatgacattacagcaa	(SEQ. ID. NO:25)

In separate experiments, a DNA chip was hybridized to each of the targets wt480 and mu480, respectively, and then scanned with a confocal microscope. FIG. 5, in panels A, B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to the wt480 target; in panel B, the chip was hybridized to the mu480 target; and in panel C, the chip was hybridized to a mixture of the wild-type and mutant targets. FIG. 6, in sheets 1-3, corresponding to panels A, B, and C of FIG. 5, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest").

These figures show that the chip could be used to sequence a 16-base stretch from the center of the target wt480 and that discrimination against mismatches is quite good throughout the sequenced region. When the DNA chip was exposed to the target mu480, only one probe in the portion of the chip shown bound the target well: the probe in the set of probes devoted to identifying the base at position 46 in exon 10 and that has an A in the position of substitution and so is fully complementary to the central portion of the mutant target. All other probes in that region of the chip have at least one mismatch with the mutant target and therefore bind much less of it. In spite of that fact, the sequence of mu480 for several positions to both sides of the mutation can be read from the chip, albeit with much-reduced intensities from those observed with the wild-type target.

The results also show that, when the two targets were mixed together and exposed to the chip, the hybridization pattern observed was a combination of the other two pat-

terns. These primers can be used to amplify exon 10 or exon 11 sequences; in another embodiment, multiplex PCR is employed, using two or more pairs of primers to amplify more than one exon at a time.

The product of amplification was then used as a template for the RNA polymerase, with fluoresceinated UTP present to label the RNA product. After sufficient RNA was made, it was fragmented and applied to an exon 10 DNA chip for 15 minutes, after which the chip was washed with hybridization buffer and scanned with the fluorescence microscope. A useful positive control included on many CF exon 10 chips is the 8-mer 3'-CGCCGCCG-5'. FIG. 7, in panels A and B, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to nucleic acid derived from the genomic DNA of an individual with wild-type Δ F508 sequences; in panel B, the target nucleic acid originated from a heterozygous (with respect to the Δ F508 mutation) individual. FIG. 8, in sheets 1 and 2, corresponding to panels A and B of FIG. 7, shows graphs of fluorescence intensity versus tiling position.

These figures show that the sequence of the wild-type RNA can be called for most of the bases near the mutation. In the case of the Δ F508 heterozygous carrier, one particular probe, the same one that distinguished so clearly between the wild-type and mutant oligonucleotide targets in the model system described above, in the T-lane binds a large amount of RNA, while the same probe binds little RNA from the wild-type individual. These results show that the DNA chips of the invention are capable of detecting the Δ F508 mutation in a heterozygous carrier.

Thus, the present invention provides methods for synthesizing large numbers of oligonucleotide probes on a glass substrate and unique probe sets in a defined array in which the probes are arranged in the array by the "tiling" method of the invention. The DNA chips produced by the method can be used to detect mutations in particular sequences of a target nucleic acid, such as genomic DNA or RNA produced from transcription of an amplified genomic DNA. These

chips can be used to detect both point mutations and small deletions. Moreover, the pattern of hybridization to the chip allows inferences to be drawn about the sequences of the mutant DNAs.

For example, in the model system involving the cystic fibrosis point mutation G480C, the A-lane probe whose position of substitution corresponds to the position of the mutation does not bind much wild-type target, because in the wild-type sequence, a G occupies that position. However, it binds mutant target very well, allowing one to infer correctly that the mutation involves a change of that G to a T. Similarly, in the case of the three-base deletion in cystic fibrosis known as $\Delta F508$, the T-lane probe that binds mutant target so intensely is responding to the fact that the deletion has brought a CAT sequence into the position occupied by a CTT sequence in the wild-type target. The DNA chips of the invention can be used to detect and sequence not only known mutations in an organism's genome but also new mutations not previously characterized. The DNA chips and methods of the invention can also be used to detect specific sequences in other CFTR exons as well as other human genes for purposes of research and clinical genetic analysis, as demonstrated below.

Detection of Specific Human Mitochondrial DNA Sequences with DNA Chips

As noted above, the present invention provides DNA chips on which a known DNA sequence is represented as an array of overlapping oligonucleotides on a solid support. This set of oligonucleotides is used to probe a target nucleic acid comprising the known sequence, allowing mutations to be detected. As also noted above, there are advantages in

some applications to using a minimal set of oligonucleotides specific to the sequence of interest, rather than a set of all possible N-mers. Some of these advantages include: (i) each position in the array is highly informative, whether or not hybridization occurs; (ii) nonspecific hybridization is minimized; (iii) it is straightforward to correlate hybridization differences with sequence differences, particularly with reference to the hybridization pattern of a known standard; and (iv) the ability to address each probe independently during synthesis, using high resolution photolithography, allows the array to be designed and optimized for any sequence. For example the length of any probe can be varied independently of the others.

The present invention illustrates these advantages by providing DNA chips and analytical methods for detecting specific sequences of human mitochondrial DNA. In one preferred embodiment, the invention provides a DNA chip for analyzing sequences contained in a 1.3 kb fragment of human mitochondrial DNA from the "D-loop" region, the most polymorphic region of human mitochondrial DNA. One such chip comprises a set of 269 overlapping oligonucleotide probes of varying length in the range of 9-14 nucleotides with varying overlaps arranged in ~600x600 micron features or synthesis sites in an array 1 cmx1 cm in size. The probes on the chip are shown in columnar form below. An illustrative mitochondrial DNA chip of the invention comprises the following probes (X, Y coordinates are shown, followed by the sequence; "DL3" represents the 3'-end of the probe, which is covalently attached to the chip surface.)

0	0	DL3AGTGGGGTATTT	(SEQ ID. NO:26)	9	2	DL3GGTAGGATGGGT	(SEQ ID. NO:67)
1	0	DL3GGGTATTTAGTT	(SEQ ID. NO:27)	10	2	DL3GGATGGGTCGTG	(SEQ ID. NO:68)
2	0	DL3TTAGTTTATCCAA	(SEQ ID. NO:28)	11	2	DL3GGTCGTGTGTGT	(SEQ ID. NO:69)
3	0	DL3ATCCAAACCAGG	(SEQ ID. NO:29)	12	2	DL3GTGTGTGTGGCG	(SEQ ID. NO:70)
4	0	DL3ACCAGGATCGGA	(SEQ ID. NO:30)	13	2	DL3GTGGCGACGAT	(SEQ ID. NO:71)
5	0	DL3CGTGTGTGTGTGG	(SEQ ID. NO:31)	14	2	DL3GACGATTGGGGT	(SEQ ID. NO:72)
6	0	DL3CGTGTGTGTGTGGC	(SEQ ID. NO:32)	15	2	DL3ATTGGGGTATGG	(SEQ ID. NO:73)
7	0	DL3TCGTGTGTGTGTGG	(SEQ ID. NO:33)	16	2	DL3GTATGGGGCTTG	(SEQ ID. NO:74)
8	0	DL3GTAGGATGGGTC	(SEQ ID. NO:34)	0	3	DL3GGATTGTGGTCG	(SEQ ID. NO:75)
9	0	DL3AGGATGGGTCGT	(SEQ ID. NO:35)	1	3	DL3TGGTCGGATTGG	(SEQ ID. NO:76)
10	0	DL3GATGGGTCGTGT	(SEQ ID. NO:36)	2	3	DL3GGATTGGTCTAAA	(SEQ ID. NO:77)
11	0	DL3TGGCGACGATTG	(SEQ ID. NO:37)	3	3	DL3TCTAAAGTTTAAA	(SEQ ID. NO:78)
12	0	DL3GCGACGATTGGG	(SEQ ID. NO:38)	4	3	DL3GTTTAAATAGAA	(SEQ ID. NO:79)
13	0	DL3TGGGGGGGA		5	3	DL3ATAGAAAAACCG	(SEQ ID. NO:80)
14	0	DL3GAGGGGGCG		6	3	DL3AGAAAAACCGC	(SEQ ID. NO:81)
15	0	DL3GGAGGGGGCGA	(SEQ ID. NO:39)	7	3	DL3AACCGCCATAC	(SEQ ID. NO:82)
16	0	DL3GAGGGGGCGA	(SEQ ID. NO:40)	8	3	DL3CCATACGTGAAAA	(SEQ ID. NO:83)
0	1	DL3GGCTTGGTTGG	(SEQ ID. NO:41)	9	3	DL3ACGTGAAAAATTGT	(SEQ ID. NO:84)
1	1	DL3GGTTGGTTTGGG	(SEQ ID. NO:42)	10	3	DL3AATTGTCAATTGG	(SEQ ID. NO:85)
2	1	DL3TGGGGTTTCTAG	(SEQ ID. NO:43)	11	3	DL3TGTCACTGGGGG	(SEQ ID. NO:86)
3	1	DL3GTTTCTAGTGGG	(SEQ ID. NO:44)	12	3	DL3TGGGGTTTGA	(SEQ ID. NO:87)
4	1	DL3AGTGGGGGGTGT	(SEQ ID. NO:45)	13	3	DL3GGGTTGATTGTGT	(SEQ ID. NO:88)
5	1	DL3GGGGTGTCAAAT	(SEQ ID. NO:46)	14	3	DL3TTGTGTAATAAAA	(SEQ ID. NO:89)
6	1	DL3GTCAAATACATCG	(SEQ ID. NO:47)	15	3	DL3AATAAAGGGGGA	(SEQ ID. NO:90)
7	1	DL3ACATCGAATGGAG	(SEQ ID. NO:48)	16	3	DL3TAAAGGGGGAGG	(SEQ ID. NO:91)
8	1	DL3CGAATGGAGGAG	(SEQ ID. NO:49)	0	4	DL3GTTTTTTAAAGG	(SEQ ID. NO:92)
9	1	DL3GAGGAGTTTCGT	(SEQ ID. NO:50)	1	4	DL3TTTTAAAGGTGG	(SEQ ID. NO:93)
10	1	DL3TTTCGTTATGTGA	(SEQ ID. NO:51)	2	4	DL3AGGTGGTTTGG	(SEQ ID. NO:94)
11	1	DL3ATGTGACTTTTAC	(SEQ ID. NO:52)	3	4	DL3TTGGGGGGGAG	(SEQ ID. NO:95)
12	1	DL3GACTTTTACAAAT	(SEQ ID. NO:53)	4	4	DL3GGAGGGGGCG	(SEQ ID. NO:96)
13	1	DL3AAATCTGCCCGA	(SEQ ID. NO:54)	5	4	DL3GGGGCGAAGAC	(SEQ ID. NO:97)
14	1	DL3AATCTGCCCGAG	(SEQ ID. NO:55)	6	4	DL3GAAGACCGGATG	(SEQ ID. NO:98)
15	1	DL3CCCGAGTGTAGT	(SEQ ID. NO:56)	7	4	DL3CCCGATGTCTGT	(SEQ ID. NO:99)
16	1	DL3AGTGTATGGGGG	(SEQ ID. NO:57)	8	4	DL3GTCTGTAATTTGT	(SEQ ID. NO:100)
0	2	DL3GGGAGGGGTGAG	(SEQ ID. NO:58)	9	4	DL3CGTGAATTTGTGT	(SEQ ID. NO:101)
1	2	DL3GGTGAGGGGTATG	(SEQ ID. NO:59)	10	4	DL3TTGTGTAGAGACG	(SEQ ID. NO:102)
2	2	DL3GGTATGATGATTAG	(SEQ ID. NO:60)	11	4	DL3TAGAGACGGTTT	(SEQ ID. NO:103)
3	2	DL3GATTAGAGTAAGT	(SEQ ID. NO:61)	12	4	DL3ACGGTTTGGGG	(SEQ ID. NO:104)
4	2	DL3TTAGAGTAAGTTA	(SEQ ID. NO:62)	13	4	DL3TGGGGTTTTTGT	(SEQ ID. NO:105)
				14	4	DL3GGGTTTTTGT	(SEQ ID. NO:106)

-continued

5	2	DL3AAGTTATGTTGGG	(SEQ ID. NO:63)	15	4	DL3TTGTTTCTTGGG	(SEQ ID. NO:107)
6	2	DL3GTTGGGGGGCG	(SEQ ID. NO:64)	16	4	DL3TCTTGGGATTGTG	(SEQ ID. NO:108)
7	2	DL3GGGGGGGGTA	(SEQ ID. NO:65)	0	5	DL3TGATGAATGATT	(SEQ ID. NO:109)
8	2	DL3GCGGGTAGGAT	(SEQ ID. NO:66)	1	5	DL3TGATTTCACACAA	(SEQ ID. NO:110)
2	5	DL3ACACAATTAATTA	(SEQ ID. NO:111)	14	7	DL3CTCTGCGACCTC	(SEQ ID. NO:157)
3	5	DL3AATTAATTACGAA	(SEQ ID. NO:112)	15	7	DL3GACCTCGGCCT	(SEQ ID. NO:158)
4	5	DL3TACGAACATCCTG	(SEQ ID. NO:113)	16	7	DL3TCGGCCTCGTG	(SEQ ID. NO:159)
5	5	DL3ACGAACATCCTGT	(SEQ ID. NO:114)	0	8	DL3GTGAAGTCCCAG	(SEQ ID. NO:160)
6	5	DL3TCCTGTATTATTA	(SEQ ID. NO:115)	1	8	DL3AGTCCCAGTATT	(SEQ ID. NO:161)
7	5	DL3GTATTATTATTGTT	(SEQ ID. NO:116)	2	8	DL3GTATTTCGGATT	(SEQ ID. NO:162)
8	5	DL3ATTGTTAAACTTA	(SEQ ID. NO:117)	3	8	DL3TCGGATTATCG	(SEQ ID. NO:163)
9	5	DL3AAACTTACAGACG	(SEQ ID. NO:118)	4	8	DL3GATTATCGGGT	(SEQ ID. NO:164)
10	5	DL3ACAGACGTGTCCG	(SEQ ID. NO:119)	5	8	DL3ATCGGGTGTGCA	(SEQ ID. NO:165)
11	5	DL3GTGTCCGTGAAA	(SEQ ID. NO:120)	6	8	DL3GTGCAAGGGGA	(SEQ ID. NO:166)
12	5	DL3GTGAAAGGTGTGT	(SEQ ID. NO:121)	7	8	DL3CAAGGGGAATT	(SEQ ID. NO:167)
13	5	DL3GGTGTGTCTGTAG	(SEQ ID. NO:122)	8	8	DL3GAATTATTCTGTA	(SEQ ID. NO:168)
14	5	DL3GTGTCTGTAGTA	(SEQ ID. NO:123)	9	8	DL3TCTGTAGTGCTAC	(SEQ ID. NO:169)
15	5	DL3GTAGTATTGTTTT	(SEQ ID. NO:124)	10	8	DL3GTAGTGCTACCT	(SEQ ID. NO:170)
16	5	DL3AGTATTGTTTTT	(SEQ ID. NO:125)	11	8	DL3GTACCTAGTAG	(SEQ ID. NO:171)
0	6	DL3CCTCGTGGGATA	(SEQ ID. NO:126)	12	8	DL3CTAGTAGTCCAGA	(SEQ ID. NO:172)
1	6	DL3TGGGATACAGCG	(SEQ ID. NO:127)	13	8	DL3TCCAGATAAGTGGG	(SEQ ID. NO:173)
2	6	DL3GATACAGCGTCAT	(SEQ ID. NO:128)	14	8	DL3AGATAGTGGGATA	(SEQ ID. NO:174)
3	6	DL3GCGTCATAGACAG	(SEQ ID. NO:129)	15	8	DL3GGGATAATTGGT	(SEQ ID. NO:175)
4	6	DL3AGACAGAACTAA	(SEQ ID. NO:130)	16	8	DL3TAATTGGTGAGTG	(SEQ ID. NO:176)
5	6	DL3CAGAACTAAGGA	(SEQ ID. NO:131)	0	9	DL3TATAGGGCGTGT	(SEQ ID. NO:177)
6	6	DL3TAAGGACGGAGT	(SEQ ID. NO:132)	1	9	DL3GGGCGGTGTTCTCA	(SEQ ID. NO:178)
7	6	DL3GACGGAGTAGGA	(SEQ ID. NO:133)	2	9	DL3GTGTTCTCAGCAT	(SEQ ID. NO:179)
8	6	DL3GTAGGATAATAAA	(SEQ ID. NO:134)	3	9	DL3TCAGATGAGAGG	(SEQ ID. NO:180)
9	6	DL3TAATAAATAGCG	(SEQ ID. NO:135)	4	9	DL3ATGAGAGGAGCG	(SEQ ID. NO:181)
10	6	DL3ATAGCGTAGGAT	(SEQ ID. NO:136)	5	9	DL3AGGAGCGAGGC	(SEQ ID. NO:182)
11	6	DL3TAGCGTAGGATG	(SEQ ID. NO:137)	6	9	DL3CGAGGCCCGCG	(SEQ ID. NO:183)
12	6	DL3AGGATGCAAGTT	(SEQ ID. NO:138)	7	9	DL3GCCCGGGTATT	(SEQ ID. NO:184)
13	6	DL3ATGCAAGTTATAA	(SEQ ID. NO:139)	8	9	DL3CGGGTATTGTGA	(SEQ ID. NO:185)
14	6	DL3GTTATAATGTCCG	(SEQ ID. NO:140)	9	9	DL3GTGAACCCCAT	(SEQ ID. NO:186)
15	6	DL3ATGTCCCGCTGT	(SEQ ID. NO:141)	10	9	DL3CCCATCGATT	(SEQ ID. NO:187)
16	6	DL3TCCGCTTGTATG	(SEQ ID. NO:142)	11	9	DL3ATCGATTTCATT	(SEQ ID. NO:188)
0	7	DL3GTGAGTGCCCTC	(SEQ ID. NO:143)	12	9	DL3TTTCACTTGACAT	(SEQ ID. NO:189)
1	7	DL3TGCCCTCGAGAG	(SEQ ID. NO:144)	13	9	DL3TTGACATAGAGT	(SEQ ID. NO:190)
2	7	DL3CCCTCGAGAGGTA	(SEQ ID. NO:145)	14	9	DL3TAGAGCTGTAGAC	(SEQ ID. NO:191)
3	7	DL3AGAGGTACGTAA	(SEQ ID. NO:146)	15	9	DL3GTAGACCAAGGA	(SEQ ID. NO:192)
4	7	DL3ACGTAAACCATA	(SEQ ID. NO:147)	16	9	DL3ACCAAGGATGAAG	(SEQ ID. NO:193)
5	7	DL3ACCATAAAGACAG	(SEQ ID. NO:148)	0	10	DL3CGTGAATGTGAG	(SEQ ID. NO:194)
6	7	DL3AAAGCAGACCC	(SEQ ID. NO:149)	1	10	DL3TGTGAGTTAGGG	(SEQ ID. NO:195)
7	7	DL3AGACCCCAT	(SEQ ID. NO:150)	2	10	DL3TCAGTTTAGGGA	(SEQ ID. NO:196)
8	7	DL3CCCCATACGT	(SEQ ID. NO:151)	3	10	DL3TAGGGAAGAGCA	(SEQ ID. NO:197)
9	7	DL3CATAGTGTGCGCT	(SEQ ID. NO:152)	4	10	DL3AAGAGCAGGGGT	(SEQ ID. NO:198)
10	7	DL3GTGCGCTATCAG	(SEQ ID. NO:153)	5	10	DL3CAGGGGTACCTA	(SEQ ID. NO:199)
11	7	DL3GCGCTATCAGTA	(SEQ ID. NO:154)	6	10	DL3GGTACCTACTGG	(SEQ ID. NO:200)
12	7	DL3TCAGTAACGCTC	(SEQ ID. NO:155)	7	10	DL3TACTGGGGGGA	(SEQ ID. NO:201)
13	7	DL3GTAACGCTCTGC	(SEQ ID. NO:156)	8	10	DL3GGGGGAGTCTAT	(SEQ ID. NO:202)
9	10	DL3AGTCTATCCCCA	(SEQ ID. NO:203)	11	13	DL3CATGATTTTGG	(SEQ ID. NO:246)
10	10	DL3ATCCCCAGGGA	(SEQ ID. NO:204)	12	13	DL3TTTTGGGTTAGG	(SEQ ID. NO:247)
11	10	DL3CAGGGAAGTGGT	(SEQ ID. NO:205)	13	13	DL3GGGTTAGGATGT	(SEQ ID. NO:248)
12	10	DL3ACTGGTGGTAGG	(SEQ ID. NO:206)	14	13	DL3GGATGTAGTTTGG	(SEQ ID. NO:249)
13	10	DL3CTGGTGGTAGGA	(SEQ ID. NO:207)	15	13	DL3GTAGTTTGGG	(SEQ ID. NO:250)
14	10	DL3GTAGGAGGCACA	(SEQ ID. NO:208)	16	13	DL3TTTGGGGAGG	(SEQ ID. NO:251)
15	10	DL3GGCACATTTAGT	(SEQ ID. NO:209)	5	14	DL3GGGTTTATACTG	(SEQ ID. NO:252)
16	10	DL3TTTAGTTATAGGG	(SEQ ID. NO:210)	6	14	DL3ATAACTGAGTGGG	(SEQ ID. NO:253)
0	11	DL3AGGTTTACGGTG	(SEQ ID. NO:211)	7	14	DL3AACTGAGTGGGT	(SEQ ID. NO:254)
1	11	DL3TACGGTGGGGA	(SEQ ID. NO:212)	8	14	DL3GTGGGTAGTTGT	(SEQ ID. NO:255)
2	11	DL3GTGGGGAGTGG	(SEQ ID. NO:213)	9	14	DL3GTAGTTGTTGGC	(SEQ ID. NO:256)
3	11	DL3GGGAGTGGGTGA	(SEQ ID. NO:214)	10	14	DL3GTTGGCGATACA	(SEQ ID. NO:257)
4	11	DL3GGGTGATCCTATG	(SEQ ID. NO:215)	11	14	DL3CGATACATAAAAG	(SEQ ID. NO:258)
5	11	DL3CCATGGTTGTTT	(SEQ ID. NO:216)	12	14	DL3TAAAGCATGTAA	(SEQ ID. NO:259)
6	11	DL3GGTGTGTTGGATG	(SEQ ID. NO:217)	13	14	DL3GCATGTAATGACG	(SEQ ID. NO:260)
7	11	DL3GTTTGGATGGGT	(SEQ ID. NO:218)	14	14	DL3ATGACGGTCCGT	(SEQ ID. NO:261)
8	11	DL3ATGGGTGGGAAT	(SEQ ID. NO:219)	15	14	DL3GTGGGTGTAAT	(SEQ ID. NO:262)
9	11	DL3GGGAATTGTGATG	(SEQ ID. NO:220)	16	14	DL3GGTACTTATAACA	(SEQ ID. NO:263)
10	11	DL3GTGATGTATCATGT	(SEQ ID. NO:221)	5	15	DL3TCGATTCTAAGAT	(SEQ ID. NO:264)
11	11	DL3TCATGTATTTCGG	(SEQ ID. NO:222)	6	15	DL3TAAGATTAAATTT	(SEQ ID. NO:265)
12	11	DL3TATTTCGGTAA	(SEQ ID. NO:223)	7	15	DL3AAATTGAATAAG	(SEQ ID. NO:266)
13	11	DL3TTCGGTAAATGG	(SEQ ID. NO:224)	8	15	DL3AATAAGAGACAAG	(SEQ ID. NO:267)
14	11	DL3GTAAATGGCATGT	(SEQ ID. NO:225)	9	15	DL3AAGAGACAAGAAA	(SEQ ID. NO:268)
15	11	DL3GCATGTAATCGTG	(SEQ ID. NO:226)	10	15	DL3AAGAAAGTACCC	(SEQ ID. NO:269)
16	11	DL3GTAATCGTGTAA	(SEQ ID. NO:227)	11	15	DL3AAAGTACCCCTT	(SEQ ID. NO:270)
5	12	DL3GGGAGGGGTAC	(SEQ ID. NO:228)	12	15	DL3CCCTTCGTCTA	(SEQ ID. NO:271)
6	12	DL3GGGTACGAATGT	(SEQ ID. NO:229)	13	15	DL3CTTCGTCTAAAC	(SEQ ID. NO:272)
7	12	DL3ACGAATGTTCTGT	(SEQ ID. NO:230)	14	15	DL3CTAAACCCATGG	(SEQ ID. NO:273)
8	12	DL3GTTCGTTCATGT	(SEQ ID. NO:231)	15	15	DL3AACCCATGGTGG	(SEQ ID. NO:274)
9	12	DL3CGTTCATGTCGTT	(SEQ ID. NO:232)	16	15	DL3TGGTGGGTTTCA	(SEQ ID. NO:275)

-continued

10	12	DL3GTCGTTAGTTGG	(SEQ ID. NO:233)	5	16	DL3TTGGAAAAAGGT	(SEQ ID. NO:276)
11	12	DL3TAGTTGGGAGTT	(SEQ ID. NO:234)	6	16	DL3AAAAGGTTCTCTG	(SEQ ID. NO:277)
12	12	DL3GGAGTTGATAGTG	(SEQ ID. NO:235)	7	16	DL3GGTTCTCTGTTTA	(SEQ ID. NO:278)
13	12	DL3ATAGTGTGTAGTT	(SEQ ID. NO:236)	8	16	DL3CCTGTTTAGTCTC	(SEQ ID. NO:279)
14	12	DL3GTTAGTTGACGT	(SEQ ID. NO:237)	9	16	DL3TAGTCTCTTTT	(SEQ ID. NO:280)
15	12	DL3TGACGTTGAGGT	(SEQ ID. NO:238)	10	16	DL3CTTTTCAGAAAT	(SEQ ID. NO:281)
16	12	DL3CGTTGAGGTTTA	(SEQ ID. NO:239)	11	16	DL3AGAAATTGAGGTG	(SEQ ID. NO:282)
5	13	DL3TATAACATGCCAT	(SEQ ID. NO:240)	12	16	DL3AAATTGAGGTGGT	(SEQ ID. NO:283)
6	13	DL3AACATGCCATGGT	(SEQ ID. NO:241)	13	16	DL3GGTGGAATCGT	(SEQ ID. NO:284)
7	13	DL3CCATGGTATTAT	(SEQ ID. NO:242)	14	16	DL3TAATCGTGGGTT	(SEQ ID. NO:285)
8	13	DL3ATTATGAACCTGG	(SEQ ID. NO:243)	15	16	DL3GTGGGTTTCGAT	(SEQ ID. NO:286)
9	13	DL3AACTGGTGGACAT	(SEQ ID. NO:244)	16	16	DL3GGTTTCGATTCT	(SEQ ID. NO:287)
10	13	DL3TGGACATCATGTA	(SEQ ID. NO:245)				

No probes were present in positions X, Y=0, 12 to X, Y=4, 12; X, Y=0, 13 to X, Y=4, 13; X, Y=0, 14 to X, Y=4, 14; X, Y=0, 15 to X, Y=4, 15; X, Y=0, 16 to X, Y=4, 16; The length of each of the probes on the chip was variable to minimize differences in melting temperature and potential for cross-hybridization. Each position in the sequence is represented by at least one probe and most positions are represented by 2 or more probes. As noted above, the amount of overlap between the oligonucleotides varies from probe to probe. FIG. 9 shows the human mitochondrial genome; "O_H" is the H strand origin of replication, and arrows indicate the cloned unshaded sequence.

DNA was prepared from hair roots of six human donors (mt1 to mt6) and then amplified by PCR and cloned into M13; the resulting clones were sequenced using chain terminators to verify that the desired specific sequences were present. DNA from the sequenced M13 clones was amplified by PCR, transcribed in vitro, and labeled with fluorescein-UTP using T3 RNA polymerase. The 1.3 kb RNA transcripts were fragmented and hybridized to the chip. The results showed that each different individual had DNA that produced a unique hybridization fingerprint on the chip and that the differences in the observed patterns could be correlated with differences in the cloned genomic DNA sequence. The results also demonstrated that very long sequences of a target nucleic acid can be represented comprehensively as a specific set of overlapping oligonucleotides and that arrays of such probe sets can be usefully applied to genetic analysis.

The sample nucleic acid was hybridized to the chip in a solution composed of 6xSSPE, 0.1% Triton-X 100 for 60 minutes at 15° C. The chip was then scanned by confocal scanning fluorescence microscopy. The individual features on the chip were 588x588 microns, but the lower left 5x5 square features in the array did not contain probes. To quantitate the data, pixel counts were measured within each synthesis site. Pixels represent 50x50 microns. The fluorescence intensity for each feature was scaled to a mean determined from 27 bright features. After scanning, the chip was stripped and rehybridized; all six samples were hybridized to the same chip. FIG. 10 shows the image observed from the mt4 sample on the DNA chip. FIG. 11 shows the image observed from the mt5 sample on the DNA chip. FIG. 12 shows the predicted difference image between the mt4 and mt5 samples on the DNA chip based on mismatches between the two samples and the reference sequence (see Anderson et al., 1981, *Nature* 290: 457-465, incorporated herein by reference). FIG. 13 shows the actual difference image observed.

The results show that, in almost all cases, mismatched probe/target hybrids resulted in lower fluorescence intensity than perfectly matched hybrids. Nonetheless, some probes detected mutations (or specific sequences) better than others,

and in several cases, the differences were within noise levels. Improvements can be realized by increasing the amount of overlap between probes and hence overall probe density and, for duplex DNA targets, using a second set of probes, either on the same or a separate chip, corresponding to the second strand of the target. FIG. 14, in sheets 1 and 2, shows a plot of normalized intensities across rows 10 and 11 of the array and a tabulation of the mutations detected.

FIG. 15 shows the discrimination between wild-type and mutant hybrids obtained with this chip. The median of the six normalized hybridization scores for each probe was taken. The graph plots the ratio of the median score to the normalized hybridization score versus mean counts. On this graph, a ratio of 1.6 and mean counts above 50 yield no false positives, and while it is clear that detection of some mutants can be improved, excellent discrimination is achieved, considering the small size of the array. FIG. 16 illustrates how the identity of the base mismatch may influence the ability to discriminate mutant and wild-type sequences more than the position of the mismatch within an oligonucleotide probe. The mismatch position is expressed as % of probe length from the 3'-end. The base change is indicated on the graph. These results show that the DNA chip increases the capacity of the standard reverse dot blot format by orders of magnitude, extending the power of that approach many fold and that the methods of the invention are more efficient and easier to automate than gel-based methods of nucleic acid sequence and mutation analysis.

These advantages become more apparent as chips with more and more probes are employed. To illustrate, the present invention provides a DNA chip for analyzing human mitochondrial DNA (mtDNA) that "tiles" through 648 nucleotides of human H strand mtDNA from positions 16280 to 356. The probes in the array are 15 nucleotides in length, and each position in the target sequence is represented by a set of 4 probes (A, C, G, T substitutions), which differed from one another at position 7 from the 3'-end. The array consists of 13 blocks of 4x50 probes: each block scans through 50 nucleotides of contiguous mtDNA sequence. The blocks are separated by blank rows. The 4 corner columns contain control probes; there are a total of 2600 probes in a 1.28 cmx1.28 cm square area (feature), and each area is 256x197 microns.

Labeled RNA target DNA was prepared by PCR amplification of a 1.3 kb region of human mtDNA spanning positions 15935 to 667, cloning into M13 (sequence verification was performed), and reamplification of the cloned sequences using primers tagged with T3 and T7 RNA polymerase promoter sequences and in vitro transcription to produce fluorescein-UTP labeled RNA. The RNA was fragmented and hybridized to the oligonucleotide array in a solution composed of 6xSSPE, 0.1% Triton X-100 for 60 minutes at 18° C. Unhybridized material was washed away with buffer, and the chip was scanned at 25 micron pixel resolution.

FIG. 17 provides a 5' to 3' sequence listing of one target corresponding to the probes on the chip. X is a control probe. Positions that differ in the target (i.e., are mismatched with the probe at the designated site) are in bold. FIG. 18 shows the fluorescence image produced by scanning the chip when hybridized to this sample. About 95% of the sequence could be read correctly from only one strand of the original duplex target nucleic acid. Although some probes did not provide excellent discrimination and some probes did not appear to hybridize to the target efficiently, excellent results were achieved. The target sequence differed from the probe set at six positions: 4 transitions and 2 insertions. All 4 transitions were detected, and specific probes could readily be incorporated into the array to detect insertions or deletions. FIG. 19 illustrates the detection of 4 transitions in the target sequence relative to the wild-type probes on the chip.

These results illustrate that longer sequences can be read using the DNA chips and methods of the invention, as compared to conventional sequencing methods, where reading length is limited by the resolution of gel electrophoresis. Similar results were observed when genomic DNA samples were prepared from human hair roots. Hybridization and signal detection require less than an hour and can be readily shortened by appropriate choice of buffers, temperatures, probes, and reagents. In principle, longer sequence reads can be obtained than by conventional sequencing, where reading length is limited by the resolution of gel electrophoresis.

P53 Sequencing and Diagnostic DNA Chips

P53 is a tumor suppressor gene that has been found to be mutated in most forms of cancer (see Levine et al., 1991, *Nature* 351: 453-456, and Hollstein et al., 1991, *Science* 253: 49-53, each of which is incorporated herein by reference). In addition, there is a hereditary syndrome, Li-Fraumeni, in which individuals inherit mutant alleles of p53 and tend to have cancer at relatively young ages (Frebourg et al., 1992, *PNAS* 89: 6413-6417, incorporated herein by reference). During the development of a cancer, p53 is inactivated. The course of p53 inactivation generally involves a mutation in one copy of p53 and is often followed by deletion of the other copy. After p53 is inactivated, chromosomal abnormalities begin to appear in tumors. In the best understood form of cancer, colorectal cancer, well over 50%, perhaps 80%, of all patients with tumors have p53 mutations. In addition, p53 mutations have been found in a high proportion of lung, breast, and other tumors (Rodrigues et al., 1990, *PNAS* 87: 7555-7559, incorporated herein by reference). According to data presented by David Sidransky (1992 San Diego Conference), over 400 mutations in p53 are known.

The p53 gene spans 20 kbp in humans and has 11 exons, 10 of which are protein coding (see Tominaga et al., 1992, *Critical Reviews in Oncogenesis* 3: 257-282, incorporated herein by reference). The gene produces a 53 kilodalton phosphoprotein that regulates DNA replication. The protein acts to halt replication at the G1/S boundary in the cell cycle and is believed to act as a "molecular policeman," shutting down replication when the DNA is damaged or blocking the reproduction of DNA viruses (see Lane, 1992, *Nature* 358: 15-16, incorporated herein by reference). There is substantial interest in the cancer research community in analyzing p53 mutations. The NCI is currently funding contracts to characterize the p53 mutation spectra caused by various carcinogens. In addition, there are research projects which involve sequencing p53 from spontaneously arising tumors. A major resource in these studies is the huge supply of biopsy material stored in paraffin blocks. Also, there are projects which are aimed at analyzing the relationship

between the particular mutation in p53 and the functioning of the resulting protein. Furthermore, there are projects looking at the germline inheritance of p53 mutations and the development of cancer. The present invention provides useful DNA chips and methods for such studies.

In addition, the present invention also provides a diagnostic test kit and method and p53 probes immobilized on a DNA chip in an organized array. Currently available diagnostic tests for cancer typically have a sensitivity of about 50%. The present invention provides significant advantages over such tests, and in one embodiment provides a method for detecting cancer-causing mutations in p53 that involves the steps of (1) obtaining a biopsy, which is optionally fractionated by cryostat sectioning to enrich tumor cells to about 80% of the total cell population. The DNA or RNA is then extracted, amplified, and analyzed with a DNA chip for the presence of p53 mutations correlated with malignancy.

To illustrate the value of the DNA chips of the present invention in such a method, a DNA chip was synthesized by the VLSIPS™ method to provide an array of overlapping probes which represent or tile across a 60 base region of exon 6 of the p53 gene. To demonstrate the ability to detect substitution mutations in the target, twelve different single substitution mutations (wild type and three different substitutions at each of three positions) were represented on the chip along with the wild type. Each of these mutations was represented by a series of twelve 12-mer oligonucleotide probes, which were complementary to the wild type target except at the one substituted base. Each of the twelve probes was complementary to a different region of the target and contained the mutated base at a different position, e.g., if the substitution was at base 32, the set of probes would be complementary—with the exception of base 32—to regions of the target 21-32, 22-33, and 32-43. This enabled investigation of the effect of the substitution position within the probe. The alignment of some of the probes with a 12-mer model target nucleic acid is shown in FIG. 20.

To demonstrate the effect of probe length, an additional series of ten 10-mer probes was included for each mutation (see FIG. 21). In the vicinity of the substituted positions, the wild-type sequence was represented by every possible overlapping 12-mer and 10-mer probe. To simplify comparisons, the probes corresponding to each varied position were arranged on the chip in the rectangular regions with the following structure: each row of cells represents one substitution, with the top row representing the wild type. Each column contains probes complementary to the same region of the target, with probes complementary to the 3'-end of the target on the left and probes complementary to the 5'-end of the target on the right. The difference between two adjacent columns is a single base shift in the positioning of the probes. Whenever possible, the series of 10-mer probes were placed in four rows immediately underneath and aligned with the 4 rows of 12-mer probes for the same mutation.

To provide model targets, 5' fluoresceinated 12-mers containing all possible substitutions in the first position of codon 192 were synthesized (see the starred position in the target in FIG. 20). Solutions containing 10 nM target DNA in 6xSSPE, 0.25% Triton X-100 were hybridized to the chip at room temperature for several hours. While target nucleic acid was hybridized to the chip, the fluorophores on the chip were excited by light from an argon laser, and the chip was scanned with an autofocus confocal microscope. The emitted signals were processed by a PC to produce an image using image analysis software. By 1 to 3 hours, the signal had reached a plateau; to remove the hybridized target and

allow hybridization to another target, the chip was stripped with 60% formamide, 2xSSPE at 17° C. for 5 minutes. The washing buffer and temperature can vary, but the buffer typically contains 2-to-3xSSPE, 10-to-60% formamide (one can use multiple washes, increasing the formamide concentration by 10% each wash, and scanning between washes to determine when the wash is complete), and optionally a small percentage of Triton X-100, and the temperature is typically in the range of 15° to 18° C.

Very distinct patterns were observed after hybridization with targets with 1 base substitutions and visualization with a confocal microscope and software analysis, as shown in FIG. 22. In general, the probes which form perfect matches with the target retain the highest signal. For example, in the first image in Figure PC, the 12-mer probes that form perfect matches with the wild-type (WT) target are in the first row (top). The 12-mer probes with single base mismatches are located in the second, third, and fourth rows and have much lower signals. The data is also depicted graphically in FIG. 23. On each graph, the X ordinate is the position of the probe in its row on the chip, and the Y ordinate is the signal at that probe site after hybridization.

When a target with a different one base substitution is hybridized the complementary set of probes has the highest signal (see pictures 2, 3, and 4 in FIG. 22 and graphs 2, 3, and 4 in FIG. 23). In each case, the probe set with no mismatches with the target has the highest signals. Within a 12-mer probe set, the signal was highest at position 6 or 7. The graphs show that the signal difference between 12-mer probes at the same X ordinate tended to be greatest at positions 5 and 8 when the target and the complementary probes formed 10 base pairs and 11 base pairs, respectively. Because tumors often have both WT and mutant p53 genes, mixed target populations were also hybridized to the chip, as shown in FIG. 24. When the hybridization solution consisted of a 1:1 mixture of WT 12-mer and a 12-mer with a substitution in position 7 of the target, the sets of probes that were perfectly matched to both targets showed higher signals than the other probe sets.

The hybridization efficiency of a 10-mer probe array as compared to a 12-mer probe array was also compared. The 10-mer and 12-mer probe arrays gave comparable signals (see graphs 1-4 in FIG. 23 and graphs 1-4 in FIG. 25). However, the 10-mer probe sets, which are in rows 5-8 (see images in FIG. 22), seemed to be better in this model system than the 12-mer probe sets at resolving one target from another, consistent with the expectation that one base mismatches are more destabilizing for 10-mers than 12-mers. Hybridization results within probe sets perfectly matched to target also followed the expectation that, the more matches the individual probe formed with the target, the higher the signal. However, duplexes with two 3' dangles (see FIG. 23, position 6 in graphs 1-4) have about as much signal as the probes which are matched along their entire length (see FIG. 23, position 7, in graphs 1-4).

This illustrative model system shows that 12-mer targets that differ by one base substitutions can be readily distinguished from one another by the novel probe array provided by the invention and that resolution of the different 12-mer targets was somewhat better with the 10-mer probe sets than with the 12-mer probe sets. The value of having several overlapping probes hybridizing to a target demonstrates the value of the multiple hybridization events that take place on a DNA chip of the invention. The results also demonstrate the feasibility of constructing a probe set to sequence the entire 1.4 kbp protein coding region of p53 or alternatively the 0.6 kbp of exons 5-9 containing mutation hot spots.

For sequencing, the p53 DNA can be cloned from the sample or directly amplified from genomic DNA by PCR. If genomic PCR is used, then the DNA can be diluted prior to amplification so that a single copy of the gene is amplified. For diagnostic purposes, the genomic DNA can be isolated from a tumor biopsy in which the tumor cells may be the majority population. As noted above, the proportion of tumor cells in a sample can be enriched by cryostat sectioning. DNA can also be isolated and amplified from tumor samples stored in paraffin blocks.

The p53 DNA in the sample can be amplified by PCR (although other amplification methods can be used) using 3-4 primer pairs generating amplicons of <3 kbp each. Illustrative primers of the invention for amplifying exon 5 of the p53 gene are shown below (B is biotin; F is fluorescein). 5'-B-CACTTGTGCCCTGACTTTCAAC-3'(SEQ. ID NO:288)

5'-F-CACTTGTGCCCTGACTTTCAAC-3'

5'-ATGCAATTAACCCTCACTAAAGGGAGACACTGTGCCCTGACTTTCAAC-3'(SEQ. ID NO:289) (has T3 promoter)

5'-B-GACCCTGGGCAACCAGCCCTGTCGT-3'(SEQ. ID NO:290)

5'-F-GACCCTGGGCAACCAGCCCTGTCGT-3'

5'-TAATACGACTCACTATAGGGAGGACCCTGGGCAACCAGCCCTGTCGT-3'(SEQ. ID NO:291) (has T3 promoter)

After PCR amplification of the target (the amplified target is called the "amplicon") one strand of the amplicon can then be isolated, i.e., using a biotinylated primer that allows capture of the undesired strand on streptavidin beads. Alternatively, asymmetric PCR can be used to generate a single-stranded target. Another approach involves the generation of single stranded RNA from the PCR product by incorporating a T7 or other RNA polymerase promoter in one of the primers. The single-stranded material can optionally be fragmented to generate smaller nucleic acids with less significant secondary structure than longer nucleic acids.

In one such method, fragmentation is combined with labeling. To illustrate, degenerate 8-mers or other degenerate short oligonucleotides are hybridized to the single-stranded target material. In the next step, a DNA polymerase is added with the four different dideoxynucleotides, each labeled with a different fluorophore. Fluorophore-labeled dideoxynucleotide are available from a variety of commercial suppliers, such as ABI. Hybridized 8-mers are extended by a labeled dideoxynucleotide. After an optional purification step, i.e., with a size exclusion column, the labeled 9-mers are hybridized to the chip. Other methods of target fragmentation can be employed. The single-stranded DNA can be fragmented by partial degradation with a DNase or partial depurination with acid. Labeling can be accomplished in a separate step, i.e., fluorophore-labeled nucleotides are incorporated before the fragmentation step or a DNA binding fluorophore, such as ethidium homodimer, is attached to the target after fragmentation.

In one embodiment, the DNA chip has an array of 10^4 to 10^5 probes tiling across the protein coding regions of p53, which comprise about 1200 bp; smaller arrays specific for the 600 bp mutational hot spot region are also useful. The probes overlap for N-2 to N-4 bases, where N is the length of the probe in bases. N is typically 10 to 14 bases long, but as will be seen below, probes 15 to 19 bases and longer are also useful. Every possible single base substitution occurring one at a time is represented in the array. The number of unique 10-mer probes with 7 base overlaps would be about

(1200/3)×4×10 or about 1.6×10^4 . To allow 3 replicates of each probe, one might have a total array size on the order of 4.8×10^4 probes. Of course, arrays of probes within the ranges of 10^2 to 10^6 probes are also useful for applications; for example, very large arrays of 10^6 or more probes are useful for sequencing or sequence checking large genomic DNA fragments. Optionally fragmented and labeled target nucleic acid hybridized to the chip is detected by a confocal microscope or other imaging device. The pattern of sites "lighting up" with target is preferably analyzed with computer assistance to provide the sequence of the target from the pattern of sites producing signals.

The invention is illustrated below with examples of DNA chips comprising very large arrays of DNA probes to "resequence" p53 target nucleic acid in a sample. To analyze DNA from exon 5 of the p53 tumor suppressor gene, a set of overlapping 17-mer probes was synthesized on a chip. The probes for the WT allele were synthesized so as to tile across the entire exon with single base overlaps between probes. For each WT probe, a sets of 4 additional probes, one for each possible base substitution at position 7, were synthesized and placed in a column relative to the WT probe. Exon 5 DNA was amplified by PCR with primers flanking the exon. One of the primers was labeled with fluorescein; the other primer was labeled with biotin. After amplification, the biotinylated strand was removed by binding to streptavidin beads. The fluoresceinated strand was used in hybridization.

About $\frac{1}{2}$ of the amplified, single-stranded nucleic acid was hybridized overnight in 5×SSPE at 60° C. to the probe chip (under a cover slip). After washing with 6×SSPE, the chip was scanned using confocal microscopy. FIG. 26 shows an image of the p53 chip hybridized to the target DNA. Analysis of the intensity data showed that 93.5% of the 184 bases of exon 5 were called in agreement with the WT sequence (see Buchman et al., 1988, *Gene* 70: 245-252, incorporated herein by reference). The miscalled bases were from positions where probe signal intensities were tied (1.6%) and where non-WT probes had the highest signal intensity (4.9%). FIG. 27 illustrates how the actual sequence was read. Gaps in the sequence of letters in the WT rows correspond to control probes or sites. Positions at which bases are miscalled are represented by letters in italic type in cells corresponding to probes in which the WT bases have been substituted by other bases.

As the diagram indicates, the miscalled bases are from the low intensity areas of the image, which may be due to secondary structure in the target or probes preventing intermolecular hybridization. To diminish the effects due to secondary structure, one can employ shorter targets (i.e., by target fragmentation) or use more stringent hybridization conditions. In addition, the use of a set of probes synthesized by tiling across the other strand of a duplex target can also provide sequence information buried in secondary structure in the other strand. It should be appreciated, however, that the pattern of low intensity areas that forms as a result of secondary structure in the target itself provides a means to identify that a specific target sequence is present in a sample. Other factors that may contribute to lower signal intensities include differences in probe densities and hybridization stabilities.

These results demonstrate the advantages provided by the DNA chips of the invention to genetic analysis. As another example, heterozygous mutations are currently sequenced by an arduous process involving cloning and repurification of DNA. The cloning step is required, because the gel sequencing systems are poor at resolving even a 1:1 mixture

of DNA. First, the target DNA is amplified by PCR with primers allowing easy ligation into a vector, which is taken up by transformation of *E. coli* which in turn must be cultured, typically on plates overnight. After growth of the bacteria, DNA is purified in a procedure that typically takes about 2 hours; then, the sequencing reactions are performed, which takes at least another hour, and the samples are run on the gel for several hours, the duration depending on the length of the fragment to be sequenced. By contrast, the present invention provides direct analysis of the PCR amplified material after brief transcription and fragmentation steps, saving days of time and labor.

An interesting clinical application for the characterization of heterozygous mutations with DNA chips is as follows. Individuals with germline cancer mutations have a very high risk for secondary tumors after treatment by irradiation. About 10% of all cancer patients may have germline mutations for p53 or other tumor suppressor genes. Thus, before deciding on a treatment modality, a physician could use the method and DNA chips of the invention to test for a germline suppressor gene mutation.

DNA Chips for Rational Therapeutic Management

The present invention also provides DNA chips that can be used by physicians to determine optimum therapeutic protocols by early, rapid detection of biologically mediated resistance to a therapeutic agent in a variety of disease states. The benefits of such DNA chips are many, as the chips will help physicians recognize health care cost savings, achieve rapid therapeutic benefits, limit administration of ineffective (due to the resistance) yet toxic drugs, monitor changes in pathogen resistance, and decrease pathogen acquisition of resistance. Important applications include the treatment of HIV, other infectious diseases, and cancer.

HIV has infected a large and expanding number of people, resulting in massive health care expenditures. HIV can rapidly become resistant to drugs used to treat the infection, primarily due to the action of the heterodimeric protein (51 kD and 66 kD) HIV reverse transcriptase (RT) encoded by the 1.7 kb pol gene. The high error rate (5-10 per round) of the RT protein is believed to account for the hypermutability of HIV. The nucleoside analogues, i.e., AZT, ddI, ddC, and d4T, commonly used to treat HIV infection are converted to nucleotide analogues by sequential phosphorylation in the cytoplasm of infected cells, where incorporation of the analogue into the viral DNA results in termination of viral replication, because the 5'→3' phosphodiester linkage cannot be completed. However, within after 6 months to 1 year of treatment, HIV typically mutates the RT gene so as to become incapable of incorporating the analogue and so resistant to treatment. Several known mutations are shown in tabular form below.

RT MUTATIONS ASSOCIATED WITH DRUG RESISTANCE

ANTI-VIRAL	CODON	aa CHANGE	nt CHANGE
AZT	67	Asp → Asn	GAC → AAC
AZT	70	Lys → Arg	AAA → AGA
AZT	215	Thr → Phe or Tyr	ACC → TTC or TAC
AZT	219	Lys → Gln or Glu	AAA → CAA or GAA
AZT	41	Met → Leu	ATG → TTG or CTG
ddI and ddC	184	Met → Val	ATG → GTG
ddI and ddC	74	Leu → Val	
TIBO 82150	100	Leu → Ile	

N.B. other mutations confer resistance to other drugs in vitro

The present invention provides DNA chips for detecting the multiple mutations in the HIV RT gene associated with

resistance to different therapeutics. These DNA chips will enable physicians to monitor mutations over time and to change therapeutics if resistance develops. The DNA chip will provide redundant confirmation of conserved HIV RT and other gene sequences, and the probes on the chip will tile through, with overlap, in important mutational hot spot regions. The chip will optionally have probes that span the entire coding region of the RT and optionally the genes for other HIV proteins, such as coat proteins. HIV target nucleic acid can be isolated from blood samples (peripheral blood lymphocytes or PBMC) and amplified by PCR, primers for which are shown in the table below.

AMPLIFICATION OF TARGET

TARGET SIZE	PRIMER 1	PRIMER 2
1,742bp	GTAGAATTCTGTGACTCAGATTGG (SEQ ID. NO:292)	GATAAGCTTGGGCCTTATCTAATCCAT (SEQ ID. NO:294)
535bp	AAATCCATACAATACTCCAGTATTGG (SEQ ID. NO:293)	ACCCATCCAAAGGAATGGAGGTTCTTTC (SEQ ID. NO:295)
323bp	Genbank/K02013 1889-1908	bases 2211-2192

The HIV RT gene chips of the invention, as well as the CF, mtDNA, and p53 DNA chips of the invention, illustrate the diverse application of the methods and probe arrays of the invention. The examples that follow describe methods for preparing nucleic acid targets from samples for application to the DNA chips of the invention and provide additional details of the methods of the invention.

EXAMPLES

I. VLSIPS™ Technology

As noted above, the VLSIPS™ technology is described in a number of patent publications and is preferred for making the oligonucleotide arrays of the invention. For completeness, a brief description of how this technology can be used to make and screen DNA chips is provided in this Example and the accompanying Figures. In the VLSIPS method, light is shone through a mask to activate functional (for oligonucleotides, typically an —OH) groups protected with a photoremovable protecting group on a surface of a solid support. After light activation, a nucleoside building block, itself protected with a photoremovable protecting group (at the 5'—OH), is coupled to the activated areas of the support. The process can be repeated, using different masks or mask orientations and building blocks, to prepare very dense arrays of many different oligonucleotide probes. The process is illustrated in FIG. 28; FIG. 29 illustrates how the process can be used to prepare "nucleoside combinatorials" or oligonucleotides synthesized by coupling all four nucleosides to form dimers, trimers, etc.

New methods for the combinatorial chemical synthesis of peptide, polycarbamate, and oligonucleotide arrays have recently been reported (see Fodor et al., 1991, *Science* 251: 767-773; Cho et al., 1993, *Science* 261: 1303-1305; and Southern et al., 1992, *Genomics* 13: 1008-10017, each of which is incorporated herein by reference). These arrays, or biological chips (see Fodor et al., 1993, *Nature* 364: 555-556, incorporated herein by reference), harbor specific chemical compounds at precise locations in a high-density, information rich format, and are a powerful tool for the study of biological recognition processes. A particularly exciting application of the array technology is in the field of DNA sequence analysis. The hybridization pattern of a DNA target to an array of shorter oligonucleotide probes is used

to gain primary structure information of the DNA target. This format has important applications in sequencing by hybridization, DNA diagnostics and in elucidating the thermodynamic parameters affecting nucleic acid recognition.

Conventional DNA sequencing technology is a laborious procedure requiring electrophoretic size separation of labeled DNA fragments. An alternative approach, termed Sequencing By Hybridization (SBH), has been proposed (Lysov et al., 1988, *Dokl. Akad. Nauk SSSR* 303: 1508-1511; Bains et al., 1988, *I. Theor. Biol.* 135: 303-307; and Drmanac et al., 1989, *Genomics* 4: 114-128, incorporated herein by reference). This method uses a set of short

oligonucleotide probes of defined sequence to search for complementary sequences on a longer target strand of DNA. The hybridization pattern is used to reconstruct the target DNA sequence. It is envisioned that hybridization analysis of large numbers of probes can be used to sequence long stretches of DNA. In immediate applications of this hybridization methodology, a small number of probes can be used to interrogate local DNA sequence.

The strategy of SBH can be illustrated by the following example. A 12-mer target DNA sequence, AGCCTAGCTGAA, (SEQ. ID NO:296) is mixed with a complete set of octanucleotide probes. If only perfect complementarity is considered, five of the 65,536 octamer probes -TCGGATCG, CGGATCGA, GGATCGAC, GATCGACT, and ATCGACTT will hybridize to the target. Alignment of the overlapping sequences from the hybridizing probes reconstructs the complement of the original 12-mer target:

```

TCGGATCG
CGGATCGA
GGATCGAC
GATCGACT
ATCGACTT
TCGGATCGACTT (SEQ. ID NO:297)

```

Hybridization methodology can be carried out by attaching target DNA to a surface. The target is interrogated with a set of oligonucleotide probes, one at a time (see Strezoska et al., 1991, *Proc. Natl. Acad. Sci. USA* 88: 10089-10093, and Drmanac et al., 1993, *Science* 260: 1649-1652, each of which is incorporated herein by reference). This approach can be implemented with well established methods of immobilization and hybridization detection, but involves a large number of manipulations. For example, to probe a sequence utilizing a full set of octanucleotides, tens of thousands, of hybridization reactions must be performed. Alternatively, SBH can be carried out by attaching probes to a surface in an array format where the identity of the probes at each site is known. The target DNA is then added to the array of probes. The hybridization pattern determined in a single experiment directly reveals the identity of all complementary probes.

As noted above, a preferred method of oligonucleotide probe array synthesis involves the use of light to direct the synthesis of oligonucleotide probes in high-density, miniaturized arrays. Photolabile 5'-protected N-acyl-deoxynucleoside phosphoramidites, surface linker chemistry, and versatile combinatorial synthesis strategies have been developed for this technology. Matrices of spatially-defined oligonucleotide probes have been generated, and the ability to use these arrays to identify complementary sequences has been demonstrated by hybridizing fluorescent labeled oligonucleotides to the DNA chips produced by the methods. The hybridization pattern demonstrates a high degree of base specificity and reveals the sequence of oligonucleotide targets.

The basic strategy for light-directed oligonucleotide synthesis (1) is outlined in FIG. 28. The surface of a solid support modified with photolabile protecting groups (X) is illuminated through a photolithographic mask, yielding reactive hydroxyl groups in the illuminated regions. A 3'-O-phosphoramidite activated deoxynucleoside (protected at the 5'-hydroxyl with a photolabile group) is then presented to the surface and coupling occurs at sites that were exposed to light. Following capping, and oxidation, the substrate is rinsed and the surface illuminated through a second mask, to expose additional hydroxyl groups for coupling. A second 5'-protected, 3'-O-phosphoramidite activated deoxynucleoside is presented to the surface. The selective photodeprotection and coupling cycles are repeated until the desired set of products is obtained.

Light directed chemical synthesis lends itself to highly efficient synthesis strategies which will generate a maximum number of compounds in a minimum number of chemical steps. For example, the complete set of 4n polynucleotides (length n), or any subset of this set can be produced in only 4xn chemical steps. See FIG. 29. The patterns of illumination and the order of chemical reactants ultimately define the products and their locations. Because photolithography is used, the process can be miniaturized to generate high-density arrays of oligonucleotide probes. For an example of the nomenclature useful for describing such arrays, an array containing all possible octanucleotides of dA and dT is written as (A+T)⁸. Expansion of this polynomial reveals the identity of all 256 octanucleotide probes from AAAAAAAAAA to TTTTTTTT. A DNA array composed of complete sets of dinucleotides is referred to as having a complexity of 2. The array given by (A+T+C+G)⁸ is the full 65,536 octanucleotide array of complexity four.

To carry out hybridization of DNA targets to the probe arrays, the arrays are mounted in a thermostatically controlled hybridization chamber. Fluorescein labeled DNA targets are injected into the chamber and hybridization is allowed to proceed for ¼ to 2 hours. The surface of the matrix is scanned in an epifluorescence microscope (Zeiss Axioscop 20) equipped with photon counting electronics using 50–100 μW of 488 nm excitation from an Argon ion laser (Spectra Physics model 2020). All measurements are acquired with the target solution in contact with the probe matrix. Photon counts are stored and image files are presented after conversion to an eight bit image format. See FIG. 33.

When hybridizing a DNA target to an oligonucleotide array, N=Lt-(Lp-1) complementary hybrids are expected, where N is the number of hybrids, Lt is the length of the DNA target, and Lp is the length of the oligonucleotide probes on the array. For example, for an 11-mer hybridized to an octanucleotide array, N=4. Hybridizations with mismatches at positions that are 2 to 3 residues from either end

of the probes will generate detectable signals. Modifying the above expression for N, one arrives at a relationship estimating the number of detectable hybridizations (Nd) for a DNA target of length Lt and an array of complexity C. Assuming an average of 5 positions giving signals above background: $Nd = (1+5(C-1))(Lt-(Lp-1))$.

Arrays of oligonucleotides can be efficiently generated by light-directed synthesis and can be used to determine the identity of DNA target sequences. Because combinatorial strategies are used, the number of compounds increases exponentially while the number of chemical coupling cycles increases only linearly. For example, expanding the synthesis to the complete set of 4⁸ (65,536) octanucleotides will add only four hours to the synthesis for the 16 additional cycles. Furthermore, combinatorial synthesis strategies can be implemented to generate arrays of any desired composition. For example, because the entire set of dodecamers (4¹²) can be produced in 48 photolysis and coupling cycles (bⁿ compounds requires bxn cycles), any subset of the dodecamers (including any subset of shorter oligonucleotides) can be constructed with the correct lithographic mask design in 48 or fewer chemical coupling steps. In addition, the number of compounds in an array is limited only by the density of synthesis sites and the overall array size. Recent experiments have demonstrated hybridization to probes synthesized in 25 μm sites. At this resolution, the entire set of 65,536 octanucleotides can be placed in an array measuring 0.64 cm square, and the set of 1,048,576 dodecanucleotides requires only a 2.56 cm array.

Genome sequencing projects will ultimately be limited by DNA sequencing technologies. Current sequencing methodologies are highly reliant on complex procedures and require substantial manual effort. Sequencing by hybridization has the potential for transforming many of the manual efforts into more efficient and automated formats. Light-directed synthesis is an efficient means for large scale production of miniaturized arrays for SBH. The oligonucleotide arrays are not limited to primary sequencing applications. Because single base changes cause multiple changes in the hybridization pattern, the oligonucleotide arrays provide a powerful means to check the accuracy of previously elucidated DNA sequence, or to scan for changes within a sequence. In the case of octanucleotides, a single base change in the target DNA results in the loss of eight complements, and generates eight new complements. Matching of hybridization patterns may be useful in resolving sequencing ambiguities from standard gel techniques, or for rapidly detecting DNA mutational events. The potentially very high information content of light-directed oligonucleotide arrays will change genetic diagnostic testing. Sequence comparisons of hundreds to thousands of different genes will be assayed simultaneously instead of the current one, or few at a time format. Custom arrays can also be constructed to contain genetic markers for the rapid identification of a wide variety of pathogenic organisms.

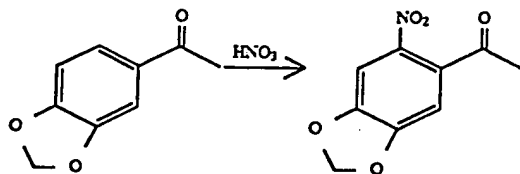
Oligonucleotide arrays can also be applied to study the sequence specificity of RNA or protein-DNA interactions. Experiments can be designed to elucidate specificity rules of non Watson-Crick oligonucleotide structures or to investigate the use of novel synthetic nucleoside analogs for antisense or triple helix applications. Suitably protected RNA monomers may be employed for RNA synthesis. The oligonucleotide arrays should find broad application deducing the thermodynamic and kinetic rules governing formation and stability of oligonucleotide complexes.

Other than the use of photoremovable protecting groups, the nucleoside coupling chemistry is very similar to that

29

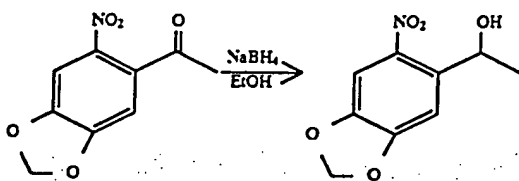
used routinely today for oligonucleotide synthesis. FIG. 30 shows the deprotection, coupling, and oxidation steps of a solid phase DNA synthesis method. FIG. 31 shows an illustrative synthesis route for the nucleoside building blocks used in the method. FIG. 32 shows a preferred photoremovable protecting group, MeNPOC, and how to prepare the group in active form. The procedures described below show how to prepare these reagents. The nucleoside building blocks are 5'-MeNPOC-THYMIDINE-3'-OCEP; 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYCYTIDINE-3'-OCEP; 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYGUANOSINE-3'-OCEP; and 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYADENOSINE-3'-OCEP.

A. Preparation of 4, 5-methylenedioxy-2-nitroacetophenone



A solution of 50 g (0.305 mole) 3,4-methylenedioxyacetophenone (Aldrich) in 200 mL glacial acetic acid was added dropwise over 30 minutes to 700 mL of cold (2-4° C.) 70% HNO₃ with stirring (NOTE: the reaction will overheat without external cooling from an ice bath, which can be dangerous and lead to side products). At temperatures below 0° C., however, the reaction can be sluggish. A temperature of 3°-5° C. seems to be optimal). The mixture was left stirring for another 60 minutes at 3°-5° C., and then allowed to approach ambient temperature. Analysis by TLC (25% EtOAc in hexane) indicated complete conversion of the starting material within 1-2 hr. When the reaction was complete, the mixture was poured into ~3 liters of crushed ice, and the resulting yellow solid was filtered off, washed with water and then suction-dried. Yield ~53 g (84%), used without further purification.

B. Preparation of 1-(4,5-Methylenedioxy-2-nitrophenyl) ethanol

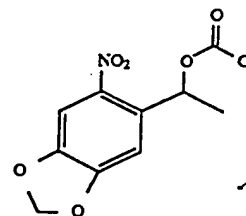
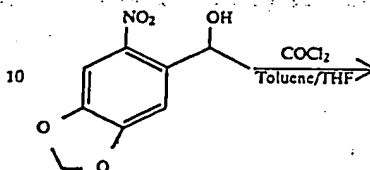


Sodium borohydride (10 g; 0.27 mol) was added slowly to a cold, stirring suspension of 53 g (0.25 mol) of 4,5-methylenedioxy-2-nitroacetophenone in 400 mL methanol. The temperature was kept below 10° C. by slow addition of the NaBH₄ and external cooling with an ice bath. Stirring was continued at ambient temperature for another two hours, at which time TLC (CH₂Cl₂) indicated complete conversion of the ketone. The mixture was poured into one liter of ice-water and the resulting suspension was neutralized with ammonium chloride and then extracted three times with 400 mL CH₂Cl₂ or EtOAc (the product can be collected by filtration and washed at this point, but it is somewhat soluble in water and this results in a yield of only ~60%). The combined organic extracts were washed with brine, then dried with MgSO₄ and evaporated. The crude product was purified from the main byproduct by dissolving it in a

30

minimum volume of CH₂Cl₂ or THF (~175 ml) and then precipitating it by slowly adding hexane (1000 ml) while stirring (yield 51 g; 80% overall). It can also be recrystallized (eg., toluene-hexane), but this reduces the yield.

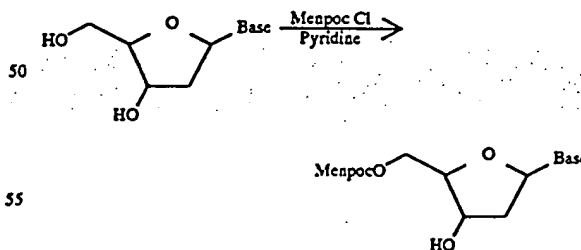
C. Preparation of 1-(4,5-methylenedioxy-2-nitrophenyl) ethyl chloroformate (MeNPOC-Cl)



Phosgene (500 mL of 20% w/v in toluene from Fluka; 965 mmole; 4 eq.) was added slowly to a cold, stirring solution of 50 g (237 mmole; 1 eq.) of 1-(4,5-methylenedioxy-2-nitrophenyl) ethanol in 400 mL dry THF. The solution was stirred overnight at ambient temperature at which point TLC (20% Et₂O/hexane) indicated >95% conversion. The mixture was evaporated (an oil-less pump with downstream aqueous NaOH trap is recommended to remove the excess phosgene) to afford a viscous brown oil. Purification was effected by flash chromatography on a short (9x13 cm) column of silica gel eluted with 20% Et₂O/hexane. Typically 55 g (85%) of the solid yellow MeNPOC-Cl is obtained by this procedure. The crude material has also been recrystallized in 2-3 crops from 1:1 ether/hexane. On this scale, ~100 ml is used for the first crop, with a few percent THF added to aid dissolution, and then cooling overnight at -20° C. (this procedure has not been optimized). The product should be stored dessicated at -20° C.

D. Synthesis of 5'-MeNPOC-2'-DEOXYNUCLEOSIDE-3'-(N,N-DIISOPROPYL 2-CYANOETHYL PHOSPHORAMIDITES

(1) 5'-MeNPOC-Nucleosides

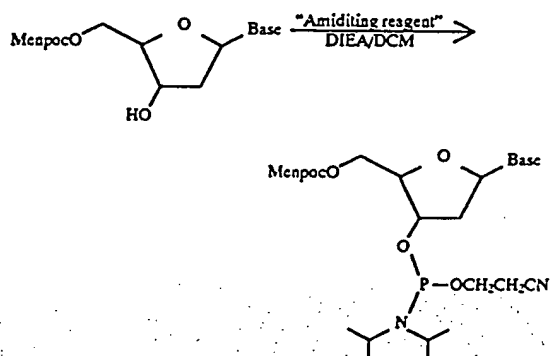


Base-THYMIDINE (T); N-4-ISOBUTYRYL 2'-DEOXYCYTIDINE (ibu-dC); N-2-PHENOXYACETYL 2'-DEOXYGUANOSINE (PAC-dG); and N-6-PHENOXYACETYL 2'-DEOXYADENOSINE (PAC-dA)

All four of the 5'-MeNPOC nucleosides were prepared from the base-protected 2'-deoxynucleosides by the following procedure. The protected 2'-deoxynucleoside (90 mmole) was dried by co-evaporating twice with 250 mL anhydrous pyridine. The nucleoside was then dissolved in

300 mL anhydrous pyridine (or 1:1 pyridine/DMF, for the dG^{3H} nucleoside) under argon and cooled to -2°C . in an ice bath. A solution of 24.6 g (90 mmole) MeNPOC-Cl in 100 mL dry THP was then added with stirring over 30 minutes. The ice bath was removed, and the solution allowed to stir overnight at room temperature (TLC: 5–10% MeOH in CH_2Cl_2 ; two diastereomers). After evaporating the solvents under vacuum, the crude material was taken up in 250 mL ethyl acetate and extracted with saturated aqueous NaHCO_3 and brine. The organic phase was then dried over Na_2SO_4 , filtered and evaporated to obtain a yellow foam. The crude products were finally purified by flash chromatography (9x30 cm silica gel column eluted with a stepped gradient of 2%–6% MeOH in CH_2Cl_2). Yields of the purified diastereomeric mixtures are in the range of 65–75%.

(2) 5'-MeNPOC-2'-DEOXYNUCLEOSIDE-3'-(N,N-DIISOPROPYL 2-CYANOETHYL PHOSPHORAMIDITES)



The four deoxynucleosides were phosphitylated using either 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite, or 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite. The following is a typical procedure. Add 16.6 g (17.4 mL; 55 mmole) of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite to a solution of 50 mmole 5'-MeNPOC-nucleoside and 4.3 g (25 mmole) diisopropylammonium tetrazolide in 250 mL dry CH_2Cl_2 under argon at ambient temperature. Continue stirring for 4–16 hours (reaction monitored by TLC: 45:45:10 hexane/ CH_2Cl_2 /Et₃N). Wash the organic phase with saturated aqueous NaHCO_3 and brine, then dry over Na_2SO_4 , and evaporate to dryness. Purify the crude amidite by flash chromatography (9x25 cm silica gel column eluted with hexane/ CH_2Cl_2 /TEA 45:45:10 for A, C, T; or 0:90:10 for G). The yield of purified amidite is about 90%.

II. PREPARATION OF LABELED DNA/HYBRIDIZATION TO ARRAY

1) PCR

PCR amplification reactions are typically conducted in a mixture composed of per reaction: 1 μL genomic DNA; 10 μL each primer (10 pmol/ μL stocks); 10 μL 10xPCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl_2); 10 μL 2 mM dNTPs (made from 100 mM dNTP stocks); 2.5 U Taq polymerase (Perkin Elmer AmpliTaqTM, 5 U/ μL); and H_2O to 100 μL . The cycling conditions are usually 40 cycles (94 $^{\circ}\text{C}$. 45 sec, 55 $^{\circ}\text{C}$. 30 sec, 72 $^{\circ}\text{C}$. 60 sec) but may need to be varied considerably from sample type to sample type. These conditions are for 0.2 mL thin wall tubes in a Perkin Elmer 9600 thermocycler. See Perkin Elmer 1992/93 catalogue for 9600 cycle time information. Target, primer length and sequence composition, among other factors, may also affect parameters.

For products in the 200 to 1000 bp size range, check 2 μL of the reaction on a 1.5% 0.5xTBE agarose gel using an appropriate size standard (phiX174 cut with HaeIII is convenient). The PCR reaction should yield several picomoles of product. It is helpful to include a negative control (i.e., 1 μL TE instead of genomic DNA) to check for possible contamination. To avoid contamination, keep PCR products from previous experiments away from later reactions, using filter tips as appropriate. Using a set of working solutions and storing master solutions separately is helpful, so long as one does not contaminate the master stock solutions.

For simple amplifications of short fragments from genomic DNA it is, in general, unnecessary to optimize Mg^{2+} concentrations. A good procedure is the following: make a master mix minus enzyme; dispense the genomic DNA samples to individual tubes or reaction wells; add enzyme to the master mix; and mix and dispense the master solution to each well, using a new filter tip each time.

2) PURIFICATION

Removal of unincorporated nucleotides and primers from PCR samples can be accomplished using the Promega Magic PCR Preps DNA purification kit. One can purify the whole sample, following the instructions supplied with the kit (proceed from section IIIB, 'Sample preparation for direct purification from PCR reactions'). After elution of the PCR product in 50 μL of TE or H_2O , one centrifuges the eluate for 20 sec at 12,000 rpm in a microfuge and carefully transfers 45 μL to a new microfuge tube, avoiding any visible pellet. Resin is sometimes carried over during the elution step. This transfer prevents accidental contamination of the linear amplification reaction with 'Magic PCR' resin. Other methods, e.g. size exclusion chromatography, may also be used.

3) LINEAR AMPLIFICATION

In a 0.2 mL thin-wall PCR tube mix: 4 μL purified PCR product; 2 μL primer (10 pmol/ μL); 4 μL 10xPCR buffer; 4 μL dNTPs (2 mM dA, dC, dG, 0.1 mM dT); 4 μL 0.1 mM dUTP; 1 μL 1 mM fluorescein dUTP (Amersham RPN 2121); 1 U Taq polymerase (Perkin Elmer, 5 U/ μL); and add H_2O to 40 μL . Conduct 40 cycles (92 $^{\circ}\text{C}$. 30 sec, 55 $^{\circ}\text{C}$. 30 sec, 72 $^{\circ}\text{C}$. 90 sec) of PCR. These conditions have been used to amplify a 300 nucleotide mitochondrial DNA fragment but are generally applicable. Even in the absence of a visible product band on an agarose gel, there should still be enough product to give an easily detectable hybridization signal. If one is not treating the DNA with uracil DNA glycosylase (see Section 4), dUTP can be omitted from the reaction.

4) FRAGMENTATION

Purify the linear amplification product using the Promega Magic PCR Preps DNA purification kit, as per Section 2 above. In a 0.2 mL thin-wall PCR tube mix: 40 μL purified labeled DNA; 4 μL 10xPCR buffer; and 0.5 μL uracil DNA glycosylase (BRL 1U/ μL). Incubate the mixture 15 min at 37 $^{\circ}\text{C}$., then 10 min at 97 $^{\circ}\text{C}$.; store at -20°C . until ready to use.

5) HYBRIDIZATION SCANNING & STRIPPING

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 mL of (fragmented) DNA in hybridization buffer and mixed well. The scan is performed in the presence of the labeled target. FIG. 33 illustrates an illustrative detection system for scanning a DNA chip. A series of scans at 30 min intervals using a hybridization temperature of 25 $^{\circ}\text{C}$. yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight. Using a laser power of 50 μW and 50 μm pixels, one should obtain maximum counts in the range of hundreds to low thousands/

pixel for a new slide. When finished, the slide can be stripped using 50% formamide, rinsing well in deionized H₂O, blowing dry, and storing at room temperature.

III. PREPARATION OF LABELED RNA HYBRIDIZATION TO ARRAY

1) TAGGED PRIMERS

The primers used to amplify the target nucleic acid should have promoter sequences if one desires to produce RNA from the amplified nucleic acid. Suitable promoter sequences are shown below and include:

(1) the T3 promoter sequence:

5'-CGGAATTAACCCTCACTAAAGG (SEQ. ID NO:298)

5'-AATTAACCCTCACTAAAGGGAG; (SEQ. ID NO:299)

(2) the T7 promoter sequence:

5' TAATACGACTCACTATAGGGAG; (SEQ. ID NO:300)

and (3) the SP6 promoter sequence:

5' ATTTAGGTGACACTATAGAA. (SEQ. ID NO:301)

The desired promoter sequence is added to the 5' end of the PCR primer. It is convenient to add a different promoter to each primer of a PCR primer pair so that either strand may be transcribed from a single PCR product.

Synthesize PCR primers so as to leave the DMT group on. DMT-on purification is unnecessary for PCR but appears to be important for transcription. Add 25 μ l 0.5M NaOH to collection vial prior to collection of oligonucleotide to keep the DMT group on. Deprotect using standard chemistry—55° C. overnight is convenient.

HPLC purification is accomplished by drying down the oligonucleotides, resuspending in 1 mL 0.1M TEAA (dilute 2.0M stock in deionized water, filter through 0.2 micron filter) and filter through 0.2 micron filter. Load 0.5 mL on reverse phase HPLC (column can be a Hamilton PRP-1 semi-prep, #79426). The gradient is 0→50% CH₃CN over 25 min (program 0.2 μ mol prep. 0→50, 25 min). Pool the desired fractions, dry down, resuspend in 200 μ l 80% HAc. 30 min RT. Add 200 μ l EtOH; dry down. Resuspend in 200 μ l H₂O, plus 20 μ l NaAc pH5.5, 600 μ l EtOH. Leave 10 min on ice; centrifuge 12,000 rpm for 10 min in microfuge. Pour off supernatant. Rinse pellet with 1 mL EtOH, dry, resuspend in 200 μ l H₂O. Dry, resuspend in 200 μ l TE. Measure A260, prepare a 10 pmol/ μ l solution in TE (10 mM Tris.Cl pH 8.0, 0.1 mM EDTA). Following HPLC purification of a 42 mer, a yield in the vicinity of 15 nmol from a 0.2 μ mol scale synthesis is typical.

2) GENOMIC DNA PREPARATION

For obtaining genomic DNA from human hair, one can extract as few as 5 hairs, including hair roots. On a clean and sterile surface, one places the hair on a piece of parafilm, and after wiping a new razor blade with EtOH cutting off the roots, the roots are transferred to a 1.5 mL microfuge tube using a pair of Millipore forceps cleaned with EtOH. Add 500 μ l (10 mM Tris.Cl pH8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, 40 mM DTT, filter sterilized) to the sample. Add 1.25 μ l 20 mg/ml proteinase K (Boehringer) Incubate at 55° C. for 2 hours, vortexing once or twice. Perform 2x0.5 mL 1:1 phenol:CHCl₃ extractions. After each extraction, centrifuge 12,000 rpm 5 min in a microfuge and recover 0.4 mL supernatant. Add 35 μ l NaAc pH5.2 plus 1 mL EtOH. Place sample on ice 45 min; then centrifuge 12,000 rpm 30 min, rinse, air dry 30 min, and resuspend in 100 μ l TE.

3) PCR

PCR is performed in a mixture containing, per reaction: 1 μ l genomic DNA; 4 μ l each primer (10 pmol/ μ l stocks); 4 μ l 10 xPCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl₂); 4 μ l 2 mM dNTPs (made from 100 mM dNTP stocks); 1 U Taq polymerase (Perkin Elmer, 5 U/ μ l); H₂O to 40 μ l. About 40 cycles (94° C. 30 sec, 55° C. 30 sec, 72° C.

30 sec) are performed, but cycling conditions may need to be varied. These conditions are for 0.2 mL thin wall tubes in Perkin Elmer 9600. For products in the 200 to 1000 bp size range, check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using an appropriate size standard. For larger or smaller volumes (20–100 μ l), one can use the same amount of genomic DNA but adjust the other ingredients accordingly.

4) IN VITRO TRANSCRIPTION

Mix: 3 μ l PCR product; 4 μ l 5xbuffer; 2 μ l DTT; 2.4 μ l 10 mM rNTPs (100 mM solutions from Pharmacia); 0.48 μ l 10 mM fluorescein-UTP (Fluorescein-12-UTP, 10 mM solution, from Boehringer Mannheim); 0.5 μ l RNA polymerase (Promega T3 or T7 RNA polymerase); and add H₂O to 20 μ l. Incubate at 37° C. for 3 h. Check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using a size standard. 5xbuffer is 200 mM Tris pH 7.5, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl, and 100 mM DTT (supplied with enzyme). The PCR product needs no purification and can be added directly to the transcription mixture. A 20 μ l reaction is suggested for an initial test experiment and hybridization; a 100 μ l reaction is considered "preparative" scale (the reaction can be scaled up to obtain more target). The amount of PCR product to add is variable; typically a PCR reaction will yield several picomoles of DNA. If the PCR reaction does not produce that much target, then one should increase the amount of DNA added to the transcription reaction (as, well as optimize the PCR). The ratio of fluorescein-UTP to UTP suggested above is 1:5, but ratios from 1:3 to 1:10—all work well. One can also label with biotin-UTP and detect with streptavidin-FITC to obtain similar results as with fluorescein-UTP detection.

For nondenaturing agarose gel electrophoresis of RNA, note that the RNA band will normally migrate somewhat faster than the DNA template band, although sometimes the two bands will comigrate. The temperature of the gel can effect the migration of the RNA band. The RNA produced from in vitro transcription is quite stable and can be stored for months (at least) at -20° C. without any evidence of degradation. It can be stored in unsterilized 6xSSPE 0.1% triton X-100 at -20° C. for days (at least) and reused twice (at least) for hybridization, without taking any special precautions in preparation or during use. RNase contamination should of course be avoided. When extracting RNA from cells, it is preferable to work very rapidly and to use strongly denaturing conditions. Avoid using glassware previously contaminated with RNases. Use of new disposable plasticware (not necessarily sterilized) is preferred, as new plastic tubes, tips, etc., are essentially RNase free. Treatment with DEPC or autoclaving is typically not unnecessary.

5) FRAGMENTATION

In a 0.2 mL thin-wall PCR tube mix: 18 μ l RNA (direct from transcription reaction—no purification required); 18 μ l H₂O; and 4 μ l 1M Tris.Cl pH9.0. Incubate at 99.9° C. for 60 min. Add to 1 mL hybridization buffer and store at -20° C. until ready to use. The alkaline hydrolysis step is very reliable. The hydrolysed target can be stored at -20° C. in 6xSSPE/0.1% Triton X-100 for at least several days prior to use and can also be reused.

6) HYBRIDIZATION SCANNING, & STRIPPING

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 mL of (hydrolysed) RNA in hybridization buffer and mixed well. Incubate for 15–30 min at 18° C. Remove the hybridization solution, which can be saved for subsequent experiments. Rinse the flow cell 4–5 times with fresh changes of 6xSSPE/0.1% Triton X-100, equilibrated to 18° C. The rinses can be

performed rapidly, but it is important to empty the flow cell before each new rinse and to mix the liquid in the cell thoroughly. The scan is performed in the presence of the labeled target. A series of scans at 30 min intervals using a hybridization temperature of 25° C. yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight. Using a laser power of 50 μ W and 50 μ m pixels, one should obtain maximum counts in the range of hundreds to low thousands/pixel for a new slide. When finished, the slide can be stripped using 50% to 100% formamide at 50° C. for 30 min,

rinsing well in deionized H₂O, blowing dry, and storing at room temperature.

These conditions are illustrative and assume a probe length of ~15 nucleotides. The stripping conditions suggested are fairly severe, but some signal may remain on the slide if the washing is not stringent. Nevertheless, the counts remaining after the wash should be very low in comparison to the signal in presence of target RNA. In some cases, much gentler stripping conditions are effective. The lower the hybridization temperature and the longer the duration of hybridization, the more difficult it is to strip the slide. Longer targets may be more difficult to strip than shorter targets.

 SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1 1) NUMBER OF SEQUENCES: 360

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGCTGACGT CAGCC

15

(2) INFORMATION FOR SEQ ID NO:2:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTGCTGACAT CAGCC

15

(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTGCTGACCT CAGCC

15

(2) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

-continued

TTGCTGACTT CAGCC

15

(2) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATTAAAGAA AATATCATCT TTGGTGTTTC CTATGATGA

39

(2) INFORMATION FOR SEQ ID NO:6:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATTAAAGAA AATATCATTG GTGTTTCCTA TGATGA

36

(2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (genomic)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CATTAAAGAA AATATCATTG GTGTTTCCTA TGATGA

36

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AACACCAATG ATGAT

15

(2) INFORMATION FOR SEQ ID NO:9:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCAAAGATNA TATTT

15

(2) INFORMATION FOR SEQ ID NO:10:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCAAAGANG ATATT

15

(2) INFORMATION FOR SEQ ID NO:11:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACCAAAGXT GATAT

15

(2) INFORMATION FOR SEQ ID NO:12:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACACCAAANA TGATA

15

(2) INFORMATION FOR SEQ ID NO:13:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AACACCAANG ATGAT

15

(2) INFORMATION FOR SEQ ID NO:14:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAACACCANA GATGA

15

(2) INFORMATION FOR SEQ ID NO:15:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAAACACCA AGATG

15

(2) INFORMATION FOR SEQ ID NO:16:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGAAACACNA AAGAT

15

(2) INFORMATION FOR SEQ ID NO:17:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGGAAACANC AAAGA

15

(2) INFORMATION FOR SEQ ID NO:18:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTTCAGAGG GTAAAAATTAA G

21

(2) INFORMATION FOR SEQ ID NO:19:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTTCAGAGT GTAAAAATTAA G

21

(2) INFORMATION FOR SEQ ID NO:20:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:20:

-continued

TAATACGACT CACTATAGGG AGATGACCTA ATAATGATGG GTTT

44

(2) INFORMATION FOR SEQ ID NO:21:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TAATACGACT CACTATAGGG AGTAGTGTGA AGGGTTCATA TGC

43

(2) INFORMATION FOR SEQ ID NO:22:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTCGGAATTA ACCCTCACTA AAGGTAGTGT GAAGGGTTCA TATGC

45

(2) INFORMATION FOR SEQ ID NO:23:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TAATACGACT CACTATAGGG AGAGCATACT AAAAGTGACT CTC

43

(2) INFORMATION FOR SEQ ID NO:24:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TAATACGACT CACTATAGGG AGACATGAAT GACATTTACA GCAA

44

(2) INFORMATION FOR SEQ ID NO:25:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGGAATTAACT CCTCACTAAA GGACATGAAT GACATTTACA GCAA

44

(2) INFORMATION FOR SEQ ID NO:26:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTTATGGGGT GA

1 2

(2) INFORMATION FOR SEQ ID NO:27:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTGATTTATG GG

1 2

(2) INFORMATION FOR SEQ ID NO:28:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AACCTATTTG ATT

1 3

(2) INFORMATION FOR SEQ ID NO:29:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGACCAAACC TA

1 2

(2) INFORMATION FOR SEQ ID NO:30:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGGCTAGGAC CA

1 2

(2) INFORMATION FOR SEQ ID NO:31:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGTGTGTGTG TGC

1 3

(2) INFORMATION FOR SEQ ID NO:31:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGTGTGTGT GTGC

1 4

(2) INFORMATION FOR SEQ ID NO:32:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGTGTGTGTG TGCT

1 4

(2) INFORMATION FOR SEQ ID NO:33:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTGGGTTAGGA TG

1 2

(2) INFORMATION FOR SEQ ID NO:34:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TGCTGGGTAG GA

1 2

(2) INFORMATION FOR SEQ ID NO:35:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:36:

-continued

TGTGCTGGGT AG

12

(2) INFORMATION FOR SEQ ID NO:37:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTTAGCAGCG GT

12

(2) INFORMATION FOR SEQ ID NO:38:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGGTTAGCAG CG

12

(2) INFORMATION FOR SEQ ID NO:39:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGCGGGGGGAG G

11

(2) INFORMATION FOR SEQ ID NO:40:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AGCGGGGGGAG

10

(2) INFORMATION FOR SEQ ID NO:41:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGTTGGTTCG G

11

(2) INFORMATION FOR SEQ ID NO:42:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GGGTTTGGTT GG

12

(2) INFORMATION FOR SEQ ID NO:43:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GATCTTTGGG GT

12

(2) INFORMATION FOR SEQ ID NO:44:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGGTGATCTT TG

12

(2) INFORMATION FOR SEQ ID NO:45:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TGTGGGGGGT GA

12

(2) INFORMATION FOR SEQ ID NO:46:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TAAACTGTGG GG

12

(2) INFORMATION FOR SEQ ID NO:47:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCTACATAAA CTG

1 3

(2) INFORMATION FOR SEQ ID NO:48:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GAGGTAAGCT ACA

1 3

(2) INFORMATION FOR SEQ ID NO:49:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GAGGAGGTAA GC

1 2

(2) INFORMATION FOR SEQ ID NO:50:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TGCTTTGAGG AG

1 2

(2) INFORMATION FOR SEQ ID NO:51:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AGTGTATTGC TTT

1 3

(2) INFORMATION FOR SEQ ID NO:52:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:52:

-continued

CATTTCAGT GTA

13

(2) INFORMATION FOR SEQ ID NO:53:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TAAACATTTT CAG

13

(2) INFORMATION FOR SEQ ID NO:54:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGCCCGTCTA AA

12

(2) INFORMATION FOR SEQ ID NO:55:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GAGCCCGTCT AA

12

(2) INFORMATION FOR SEQ ID NO:56:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TGATGTGAGC CC

12

(2) INFORMATION FOR SEQ ID NO:57:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GGGGTGATGT GA

12

(2) INFORMATION FOR SEQ ID NO:58:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GAGTGGGAGG G

11

(2) INFORMATION FOR SEQ ID NO:59:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GTATGGGAGT GG

12

(2) INFORMATION FOR SEQ ID NO:60:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GATTAGTAGT ATGG

14

(2) INFORMATION FOR SEQ ID NO:61:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TGAATGAGAT TAG

13

(2) INFORMATION FOR SEQ ID NO:62:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:62:

ATTGAATGAG ATT

13

(2) INFORMATION FOR SEQ ID NO:63:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:63:

GGGTTGTATT GAA

13

(2) INFORMATION FOR SEQ ID NO:64:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GCGGGGGTTG

10

(2) INFORMATION FOR SEQ ID NO:65:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:65:

ATGGCGGGG

10

(2) INFORMATION FOR SEQ ID NO:66:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TAGGATGGGC G

11

(2) INFORMATION FOR SEQ ID NO:67:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TGGGTAGGAT GG

12

(2) INFORMATION FOR SEQ ID NO:68:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:68:

-continued

GTGCTGGGTA GG

12

(2) INFORMATION FOR SEQ ID NO:69:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TGTGTGTGCT GG

12

(2) INFORMATION FOR SEQ ID NO:70:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:70:

GCGGTGTGTG TG

12

(2) INFORMATION FOR SEQ ID NO:71:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:71:

TAGCAGCGGT GT

12

(2) INFORMATION FOR SEQ ID NO:72:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TGGGGTTAGC AG

12

(2) INFORMATION FOR SEQ ID NO:73:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GGTATGGGGT TA

12

(2) INFORMATION FOR SEQ ID NO:74:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTGGGGTA TG

1 2

(2) INFORMATION FOR SEQ ID NO:75:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:75:

GCTGGTGTTA GG

1 2

(2) INFORMATION FOR SEQ ID NO:76:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GGTTAGGCTG GT

1 2

(2) INFORMATION FOR SEQ ID NO:77:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:77:

AAATCTGGTT AGG

1 3

(2) INFORMATION FOR SEQ ID NO:78:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:78:

AAATTTGAAA TCT

1 3

(2) INFORMATION FOR SEQ ID NO:79:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:79:

AAGATAAAAT TTG

13

(2) INFORMATION FOR SEQ ID NO:80:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:80:

GCCAAAAAGA TA

12

(2) INFORMATION FOR SEQ ID NO:81:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CGCCAAAAAG A

11

(2) INFORMATION FOR SEQ ID NO:82:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CATACCGCCA A

11

(2) INFORMATION FOR SEQ ID NO:83:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:83:

AAAAGTGCAT ACC

13

(2) INFORMATION FOR SEQ ID NO:84:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:84:

-continued

TGTTAAAAGT GCA

13

(2) INFORMATION FOR SEQ ID NO:85:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:85:

GGGTGACTGT TAA

13

(2) INFORMATION FOR SEQ ID NO:86:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GGGGGTGACT GT

12

(2) INFORMATION FOR SEQ ID NO:87:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:87:

AGTTGGGGGG T

11

(2) INFORMATION FOR SEQ ID NO:88:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:88:

TGTGTTAGTT GGG

13

(2) INFORMATION FOR SEQ ID NO:89:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:89:

AAAATAATGT GTT

13

(2) INFORMATION FOR SEQ ID NO:90:

-continued

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:90:

AGGGGAAAAT AA

12

(2) INFORMATION FOR SEQ ID NO:91:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGAGGGGAAA AT

12

(2) INFORMATION FOR SEQ ID NO:92:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:92:

GGAAATTTTT TG

12

(2) INFORMATION FOR SEQ ID NO:93:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GGTGGAAATT TT

12

(2) INFORMATION FOR SEQ ID NO:94:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:94:

GGTTTGGTGG A

11

(2) INFORMATION FOR SEQ ID NO:95:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GAGGGGGGGT T

(2) INFORMATION FOR SEQ ID NO:96:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCGGGGGAGG

(2) INFORMATION FOR SEQ ID NO:97:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:97:

CAGAAGCGGG G

(2) INFORMATION FOR SEQ ID NO:98:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:98:

GTAGGCCAGA AG

(2) INFORMATION FOR SEQ ID NO:99:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GTGCTGTAGG CC

(2) INFORMATION FOR SEQ ID NO:100:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:100:

-continued

TGTTTAAGTG CTG

13

(2) INFORMATION FOR SEQ ID NO:101:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:101:

TGTGTTTAAG TGC

13

(2) INFORMATION FOR SEQ ID NO:102:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:102:

GCAGAGATGT GTT

13

(2) INFORMATION FOR SEQ ID NO:103:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TTTGGCAGAG AT

12

(2) INFORMATION FOR SEQ ID NO:104:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:104:

GGGGTTTGCC A

11

(2) INFORMATION FOR SEQ ID NO:105:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:105:

TGTTTTTGGG GT

12

(2) INFORMATION FOR SEQ ID NO:106:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TTTGTTTTTG GG

12

(2) INFORMATION FOR SEQ ID NO:107:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:107:

GGGTTCTTTG TT

12

(2) INFORMATION FOR SEQ ID NO:108:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:108:

GTGTTAGGGT TCT

13

(2) INFORMATION FOR SEQ ID NO:109:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:109:

TTTAGTAAAT ATGT

14

(2) INFORMATION FOR SEQ ID NO:110:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:110:

AACACACTTT AGT

13

(2) INFORMATION FOR SEQ ID NO:111:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:111:

AATTAATTAA CACA

14

(2) INFORMATION FOR SEQ ID NO:112:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:112:

AAGCATTAAT TAA

13

(2) INFORMATION FOR SEQ ID NO:113:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:113:

GTCCTACAAG CAT

13

(2) INFORMATION FOR SEQ ID NO:114:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:114:

TGTCTACAA GCA

13

(2) INFORMATION FOR SEQ ID NO:115:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:115:

ATTATTATGT CCT

13

(2) INFORMATION FOR SEQ ID NO:116:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:116:

-continued

TTGTTATTAT TATG

14

(2) INFORMATION FOR SEQ ID NO:117:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:117:

ATTCAAATTG TTA

13

(2) INFORMATION FOR SEQ ID NO:118:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:118:

GCAGACATTG AAA

13

(2) INFORMATION FOR SEQ ID NO:119:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:119:

GCTGTGCAGA CA

12

(2) INFORMATION FOR SEQ ID NO:120:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:120:

AAAGTGGCTG TG

12

(2) INFORMATION FOR SEQ ID NO:121:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:121:

TGTGTGAAAA GTG

13

(2) INFORMATION FOR SEQ ID NO:122:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:121:

GATGTCGTG TGG

13

(2) INFORMATION FOR SEQ ID NO:121:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:121:

ATGATGCTG TGT

13

(2) INFORMATION FOR SEQ ID NO:124:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:124:

TTTTGTTATG ATG

13

(2) INFORMATION FOR SEQ ID NO:125:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:125:

TTTTTTGTTA TGA

13

(2) INFORMATION FOR SEQ ID NO:126:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:126:

ATAGGCTGCT CC

12

(2) INFORMATION FOR SEQ ID NO:127:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:127:

GCGACATAGG GT

12

(2) INFORMATION FOR SEQ ID NO:128:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:128:

TACTGCGACA TAG

13

(2) INFORMATION FOR SEQ ID NO:129:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:129:

GACAGATACT GCG

13

(2) INFORMATION FOR SEQ ID NO:130:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:130:

AATCAAAGAC AGA

13

(2) INFORMATION FOR SEQ ID NO:131:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:131:

AGGAATCAAA GAC

13

(2) INFORMATION FOR SEQ ID NO:132:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:132:

-continued

TGAGGCAGGA AT

12

(2) INFORMATION FOR SEQ ID NO:133:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:133:

AGGATGAGGC AG

12

(2) INFORMATION FOR SEQ ID NO:134:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:134:

AAATAATAGG ATG

13

(2) INFORMATION FOR SEQ ID NO:135:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:135:

GCGATAAATA AT

12

(2) INFORMATION FOR SEQ ID NO:136:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:136:

TAGGATGCGA TA

12

(2) INFORMATION FOR SEQ ID NO:137:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:137:

GTAGGATGCG AT

12

(2) INFORMATION FOR SEQ ID NO:138:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:138:

TTGAACGTAG GA

1 2

(2) INFORMATION FOR SEQ ID NO:139:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:139:

AATATTGAAC GTA

1 3

(2) INFORMATION FOR SEQ ID NO:140:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:140:

GCCTGTAATA TTG

1 3

(2) INFORMATION FOR SEQ ID NO:141:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:141:

TGTTTCGCTG TA

1 2

(2) INFORMATION FOR SEQ ID NO:142:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:142:

GTATGTTCCG CT

1 2

(2) INFORMATION FOR SEQ ID NO:143:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:143:

CTCCCGTGAG TG

1 2

(2) INFORMATION FOR SEQ ID NO:144:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GAGAGCTCCC GT

1 2

(2) INFORMATION FOR SEQ ID NO:145:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:145:

ATGGAGAGCT CC

1 2

(2) INFORMATION FOR SEQ ID NO:146:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:146:

AATGCATGGA GA

1 2

(2) INFORMATION FOR SEQ ID NO:147:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:147:

ATACCAAATG CA

1 2

(2) INFORMATION FOR SEQ ID NO:148:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:148:

-continued

GACGAAAATA CCA

13

(2) INFORMATION FOR SEQ ID NO:149:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:149:

CCCAGACGAA A

11

(2) INFORMATION FOR SEQ ID NO:150:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:150:

TACCCCCCAG A

11

(2) INFORMATION FOR SEQ ID NO:151:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:151:

TG CATACCCCC

11

(2) INFORMATION FOR SEQ ID NO:152:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:152:

TCGCGTG CAT AC

12

(2) INFORMATION FOR SEQ ID NO:153:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GACTATCGCG TG

12

(2) INFORMATION FOR SEQ ID NO:154:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:154:

ATGACTATCG CG

1 2

(2) INFORMATION FOR SEQ ID NO:155:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:155:

CTCGCAATGA CT

1 2

(2) INFORMATION FOR SEQ ID NO:156:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:156:

CGTCTCGCAA TG

1 2

(2) INFORMATION FOR SEQ ID NO:157:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CTCCAGCGTC TC

1 2

(2) INFORMATION FOR SEQ ID NO:158:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:158:

TCCGGCTCCA G

1 1

(2) INFORMATION FOR SEQ ID NO:159:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:159:

GTGCTCCGGC T

11

(2) INFORMATION FOR SEQ ID NO:160:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:160:

GACCCTGAAG TAG

13

(2) INFORMATION FOR SEQ ID NO:161:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:161:

TTTATGACCC TGA

13

(2) INFORMATION FOR SEQ ID NO:162:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:162:

TTTAGGCTTT ATG

13

(2) INFORMATION FOR SEQ ID NO:163:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:163:

GCTATTTAGG CT

12

(2) INFORMATION FOR SEQ ID NO:164:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:164:

-continued

TGGGCTATTT AG

12

(2) INFORMATION FOR SEQ ID NO:165:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:165:

ACGTGTGGGC TA

12

(2) INFORMATION FOR SEQ ID NO:166:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:166:

ACGGGAACGT GT

12

(2) INFORMATION FOR SEQ ID NO:167:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:167:

TTTAAGGGGA AC

12

(2) INFORMATION FOR SEQ ID NO:168:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:168:

ATGTCCTATT TAAG

14

(2) INFORMATION FOR SEQ ID NO:169:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:169:

CATCGTGATG TCT

13

(2) INFORMATION FOR SEQ ID NO:170:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:170:

TCCATCGTGA TG

12

(2) INFORMATION FOR SEQ ID NO:171:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:171:

GATGATCCAT CG

12

(2) INFORMATION FOR SEQ ID NO:172:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:172:

AGACCTGATG ATC

13

(2) INFORMATION FOR SEQ ID NO:173:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:173:

GGGTGATAGA CCT

13

(2) INFORMATION FOR SEQ ID NO:174:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:174:

ATAGGGTGAT AGA

13

(2) INFORMATION FOR SEQ ID NO:175:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:175:

TGGTTAATAG GG

12

(2) INFORMATION FOR SEQ ID NO:176:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:176:

GTGAGTGGTT AAT

13

(2) INFORMATION FOR SEQ ID NO:177:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:177:

TGTGCGGGAT AT

12

(2) INFORMATION FOR SEQ ID NO:178:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:178:

ACTCTTGTGC GG

12

(2) INFORMATION FOR SEQ ID NO:179:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:179:

TAGCACTCTT GTG

13

(2) INFORMATION FOR SEQ ID NO:180:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:180:

-continued

GGAGAGTAGC ACT

13

(2) INFORMATION FOR SEQ ID NO:181:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:181:

GCGAGGAGAG TA

12

(2) INFORMATION FOR SEQ ID NO:182:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:182:

CGGAGCGAGG A

11

(2) INFORMATION FOR SEQ ID NO:183:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:183:

GGCCCGGAGC

10

(2) INFORMATION FOR SEQ ID NO:184:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:184:

TTATGGGCCC G

11

(2) INFORMATION FOR SEQ ID NO:185:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:185:

AGTGTATATGG GC

12

(2) INFORMATION FOR SEQ ID NO:186:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:186:

TACCCCAAG TG

12

(2) INFORMATION FOR SEQ ID NO:187:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:187:

TTTAGCTACC CC

12

(2) INFORMATION FOR SEQ ID NO:188:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:188:

TTCACTTTAG CTA

13

(2) INFORMATION FOR SEQ ID NO:189:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:189:

TACAGTTCAC TTT

13

(2) INFORMATION FOR SEQ ID NO:190:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:190:

TCGAGATACA GTT

13

(2) INFORMATION FOR SEQ ID NO:191:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:191:

CAGATGTCGA GAT

13

(2) INFORMATION FOR SEQ ID NO:192:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:192:

AGGAACCAGA TG

12

(2) INFORMATION FOR SEQ ID NO:193:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:193:

GAAGTAGGAA CCA

13

(2) INFORMATION FOR SEQ ID NO:194:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:194:

GACTGTAATG TGC

13

(2) INFORMATION FOR SEQ ID NO:195:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:195:

GGGATTTGAC TGT

13

(2) INFORMATION FOR SEQ ID NO:196:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:196:

-continued

AGGGATTGA CT

12

(2) INFORMATION FOR SEQ ID NO:197:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:197:

ACGAGAAGGG AT

12

(2) INFORMATION FOR SEQ ID NO:198:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:198:

TGGGGACGAG AA

12

(2) INFORMATION FOR SEQ ID NO:199:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:199:

ATCCATGGGG AC

12

(2) INFORMATION FOR SEQ ID NO:200:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:200:

GGTCATCCAT GG

12

(2) INFORMATION FOR SEQ ID NO:201:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:201:

AGGGGGGTCA T

11

(2) INFORMATION FOR SEQ ID NO:202:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:202:

TATCTGAGGG GG

1 2

(2) INFORMATION FOR SEQ ID NO:203:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:203:

ACCCCTATCT GA

1 2

(2) INFORMATION FOR SEQ ID NO:204:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:204:

AGGGACCCCT A

1 1

(2) INFORMATION FOR SEQ ID NO:205:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:205:

TGGTCAAGGG AC

1 2

(2) INFORMATION FOR SEQ ID NO:206:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:206:

GGATGGTGGT CA

1 2

(2) INFORMATION FOR SEQ ID NO:207:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:207:

AGGATGGTGG TC

12

(2) INFORMATION FOR SEQ ID NO:208:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:208:

ACACGGAGGA TG

12

(2) INFORMATION FOR SEQ ID NO:209:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:209:

TGATTTACAC GG

12

(2) INFORMATION FOR SEQ ID NO:210:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:210:

GGGATATTGA TTT

13

(2) INFORMATION FOR SEQ ID NO:211:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:211:

GTGGCATTG GA

12

(2) INFORMATION FOR SEQ ID NO:212:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:212:

-continued

AGGGGTGGCA T

11

(2) INFORMATION FOR SEQ ID NO:213:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:213:

GGTGAGGGGT G

11

(2) INFORMATION FOR SEQ ID NO:214:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:214:

AGTGGGTGAG GG

12

(2) INFORMATION FOR SEQ ID NO:215:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:215:

GTATCCTAGT GGG

13

(2) INFORMATION FOR SEQ ID NO:216:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:216:

TTTGTTGGTA TCC

13

(2) INFORMATION FOR SEQ ID NO:217:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:217:

GTAGGTTTGT TGG

13

(2) INFORMATION FOR SEQ ID NO:218:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:218:

TGGGTAGGTT TG

1 2

(2) INFORMATION FOR SEQ ID NO:219:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:219:

TAAGGGTGGG TA

1 2

(2) INFORMATION FOR SEQ ID NO:220:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:220:

GTACTGTTAA GGG

1 3

(2) INFORMATION FOR SEQ ID NO:221:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:221:

TG TACTATGT ACTG

1 4

(2) INFORMATION FOR SEQ ID NO:222:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:222:

GGCTTTATGT ACT

1 3

(2) INFORMATION FOR SEQ ID NO:223:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:223:

AAATGGCTTT AT

1 2

(2) INFORMATION FOR SEQ ID NO:224:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:224:

GGTAAATGGC TT

1 2

(2) INFORMATION FOR SEQ ID NO:225:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:225:

TGTACGGTAA ATG

1 3

(2) INFORMATION FOR SEQ ID NO:226:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:226:

GTGCTAATGT ACG

1 3

(2) INFORMATION FOR SEQ ID NO:227:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:227:

TAATGTGCTA ATG

1 3

(2) INFORMATION FOR SEQ ID NO:228:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:228:

-continued

CATGGGGAGG G

11

(2) INFORMATION FOR SEQ ID NO:229:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:229:

TGTAAGCATG GG

12

(2) INFORMATION FOR SEQ ID NO:230:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:230:

TTGCTTGTA GCA

13

(2) INFORMATION FOR SEQ ID NO:231:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:231:

TGTACTTGCT TGT

13

(2) INFORMATION FOR SEQ ID NO:232:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:232:

TTGCTGTACT TGC

13

(2) INFORMATION FOR SEQ ID NO:233:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:233:

GGTTGATTGC TG

12

(2) INFORMATION FOR SEQ ID NO:234:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:234:

TTGAGGGTTG AT

1 2

(2) INFORMATION FOR SEQ ID NO:235:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:235:

GTGATACTTG AGG

1 3

(2) INFORMATION FOR SEQ ID NO:236:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:236:

TTGATGTCTG ATA

1 3

(2) INFORMATION FOR SEQ ID NO:237:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:237:

TGCAGTTGAT GTG

1 3

(2) INFORMATION FOR SEQ ID NO:238:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:238:

TGGAGTTGCA GT

1 2

(2) INFORMATION FOR SEQ ID NO:239:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:239:

ATT TGG AGT T GC

1 2

(2) INFORMATION FOR SEQ ID NO:240:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:240:

TACCGTACAA TAT

1 3

(2) INFORMATION FOR SEQ ID NO:241:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:241:

TGGTACCGTA CAA

1 3

(2) INFORMATION FOR SEQ ID NO:242:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:242:

TATTTATGGT ACC

1 3

(2) INFORMATION FOR SEQ ID NO:243:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:243:

GGTCAAGTAT TTA

1 3

(2) INFORMATION FOR SEQ ID NO:244:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:244:

-continued

TACAGGTGGT CAA

13

(2) INFORMATION FOR SEQ ID NO:245:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:245:

ATGTACTACA GGT

13

(2) INFORMATION FOR SEQ ID NO:246:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:246:

GGTTTTTATG TAC

13

(2) INFORMATION FOR SEQ ID NO:247:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:247:

GGATTGGGTT TT

12

(2) INFORMATION FOR SEQ ID NO:248:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:248:

TGTAGGATTG GG

12

(2) INFORMATION FOR SEQ ID NO:249:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:249:

GTTTTGATGT AGG

13

(2) INFORMATION FOR SEQ ID NO:250:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:250:

GGGTTTTCAT GT

1 2

(2) INFORMATION FOR SEQ ID NO:251:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:251:

GGAGGGGGTT T

1 1

(2) INFORMATION FOR SEQ ID NO:252:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:252:

GTCAATACTT GGG

1 3

(2) INFORMATION FOR SEQ ID NO:253:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:253:

GGGTGAGTCA ATA

1 3

(2) INFORMATION FOR SEQ ID NO:254:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:254:

TGGGTGAGTC AA

1 2

(2) INFORMATION FOR SEQ ID NO:255:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:255:

T G T T G A T G G G T G

1 2

(2) INFORMATION FOR SEQ ID NO:256:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:256:

C G G T T G T T G A T G

1 2

(2) INFORMATION FOR SEQ ID NO:257:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:257:

A C A T A G C G G T T G

1 2

(2) INFORMATION FOR SEQ ID NO:258:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:258:

G A A A A T A C A T A G C

1 3

(2) INFORMATION FOR SEQ ID NO:259:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:259:

A A T G T A C G A A A A T

1 3

(2) INFORMATION FOR SEQ ID NO:260:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:260:

-continued

GCAGTAATGT ACG

13

(2) INFORMATION FOR SEQ ID NO:261:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:261:

TGGCTGGCAG TA

12

(2) INFORMATION FOR SEQ ID NO:262:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:262:

TCATGGTGGC TG

12

(2) INFORMATION FOR SEQ ID NO:263:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:263:

ACAATATTCA TGG

13

(2) INFORMATION FOR SEQ ID NO:264:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:264:

TAGAATCTTA GCT

13

(2) INFORMATION FOR SEQ ID NO:265:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:265:

TTTAAATTAG AAT

13

(2) INFORMATION FOR SEQ ID NO:266:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:266:

GAATAAGTTT AAA

13

(2) INFORMATION FOR SEQ ID NO:267:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:267:

GAACAGAGAA TAA

13

(2) INFORMATION FOR SEQ ID NO:268:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:268:

AAAGAACAGA GAA

13

(2) INFORMATION FOR SEQ ID NO:269:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:269:

CCCATGAAAG AA

12

(2) INFORMATION FOR SEQ ID NO:270:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:270:

TTCCCCATGA AA

12

(2) INFORMATION FOR SEQ ID NO:271:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:271:

ATCTGCTTCC CC

1 2

(2) INFORMATION FOR SEQ ID NO:272:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:272:

CAAATCTGCT TC

1 2

(2) INFORMATION FOR SEQ ID NO:273:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:273:

GGTACCCAAA TC

1 2

(2) INFORMATION FOR SEQ ID NO:274:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:274:

GGTGGTACCC AA

1 2

(2) INFORMATION FOR SEQ ID NO:275:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:275:

TACTTGGGTG GT

1 2

(2) INFORMATION FOR SEQ ID NO:276:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:276:

-continued

TGGAAAAAGG TT

12

(2) INFORMATION FOR SEQ ID NO:277:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:277:

GTCCTTGGAA AA

12

(2) INFORMATION FOR SEQ ID NO:278:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:278:

ATTTGTCCTT GG

12

(2) INFORMATION FOR SEQ ID NO:279:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:279:

CTCTGATTG TCC

13

(2) INFORMATION FOR SEQ ID NO:280:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:280:

TTTTTCTCTG ATT

13

(2) INFORMATION FOR SEQ ID NO:281:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:281:

TAAAGACTTT TTC

13

(2) INFORMATION FOR SEQ ID NO:282:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:282:

GTGGAGTTAA AGA

13

(2) INFORMATION FOR SEQ ID NO:283:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:283:

TGGTGGAGTT AAA

13

(2) INFORMATION FOR SEQ ID NO:284:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:284:

TGCTAATGCT GG

12

(2) INFORMATION FOR SEQ ID NO:285:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:285:

TTGGGTGCTA AT

12

(2) INFORMATION FOR SEQ ID NO:286:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:286:

TAGCTTTGGG TG

12

(2) INFORMATION FOR SEQ ID NO:287:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:287:

TCTTAGCTTT GG

12

(2) INFORMATION FOR SEQ ID NO:288:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:288:

CACTTGTGCC CTGACTTTCA AC

22

(2) INFORMATION FOR SEQ ID NO:289:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:289:

ATGCAATTAA CCCTCACTAA AGGGAGACAC TTGTGCCCTG ACTTTCAAC

49

(2) INFORMATION FOR SEQ ID NO:290:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:290:

GACCCTGGGC AACCAGCCCT GTCGT

25

(2) INFORMATION FOR SEQ ID NO:291:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:291:

TAATACGACT CACTATAGGG AGGACCCTGG GCAACCAGCC CTGTCGT

47

(2) INFORMATION FOR SEQ ID NO:292:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:292:

-continued

GTAGAATTCT GTTGACTCAG ATTGG

25

(2) INFORMATION FOR SEQ ID NO:293:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:293:

AAATCCATAC AATACTCCAG TATTTGC

27

(2) INFORMATION FOR SEQ ID NO:294:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:294:

GATAAGCTTG GGCCTTATCT ATTCCAT

27

(2) INFORMATION FOR SEQ ID NO:295:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:295:

ACCCATCCAA AGGAATGGAG GTTCTTTC

28

(2) INFORMATION FOR SEQ ID NO:296:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (oligonucleotide)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:296:

AGCCTAGCTG AA

12

(2) INFORMATION FOR SEQ ID NO:297:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (oligonucleotide)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:297:

TCGGATCGAC TT

12

(2) INFORMATION FOR SEQ ID NO:298:

-continued

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:298:

CGGAATTAAC CCTCACTAAA GG

22

(2) INFORMATION FOR SEQ ID NO:299:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:299:

AATTAACCCT CACTAAAGGG AG

22

(2) INFORMATION FOR SEQ ID NO:300:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:300:

TAATACGACT CACTATAGGG AG

22

(2) INFORMATION FOR SEQ ID NO:301:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:301:

ATTTAGGTGA CACTATAGAA

20

(2) INFORMATION FOR SEQ ID NO:302:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:302:

GATNATATT

10

(2) INFORMATION FOR SEQ ID NO:303:

- (1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:303:

AGAGATATT

10

(2) INFORMATION FOR SEQ ID NO:304:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:304:

AAGNTGATAT

10

(2) INFORMATION FOR SEQ ID NO:305:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:305:

AAANATGATA

10

(2) INFORMATION FOR SEQ ID NO:306:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:306:

CAANGATGAT

10

(2) INFORMATION FOR SEQ ID NO:307:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:307:

CCANAGATGA

10

(2) INFORMATION FOR SEQ ID NO:308:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:308:

-continued

ACCNAAGATG

10

(2) INFORMATION FOR SEQ ID NO:309:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:309:

CACNAAGAT

10

(2) INFORMATION FOR SEQ ID NO:310:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:310:

AGAAACNACA

10

(2) INFORMATION FOR SEQ ID NO:311:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:311:

ATTTATTCT GTATTG

16

(2) INFORMATION FOR SEQ ID NO:312:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:312:

CCGACTGCAG TCGTTA

16

(2) INFORMATION FOR SEQ ID NO:313:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:313:

CCGACTGCAG TCGTT

15

(2) INFORMATION FOR SEQ ID NO:314:

-continued

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:314:

CCGACTACAG TC GTT

15

(2) INFORMATION FOR SEQ ID NO:315:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:315:

CCGACTCCAG TC GTT

15

(2) INFORMATION FOR SEQ ID NO:316:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:316:

CCGACTTCAG TC GTT

15

(2) INFORMATION FOR SEQ ID NO:317:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:317:

GTAATTTCTT TTATAGTAGA AACCACAAAG GATAC

35

(2) INFORMATION FOR SEQ ID NO:318:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:318:

CATTAAAGAA AATATCATCT TTGGTGTTTC CTATG

35

(2) INFORMATION FOR SEQ ID NO:319:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:319:

CATTAAAGAA AATATCATTC GTGTTTCCTA TG

3 2

(2) INFORMATION FOR SEQ ID NO:320:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:320:

CATTAAAGAA AATATCAT

1 8

(2) INFORMATION FOR SEQ ID NO:321:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:321:

TATTAAAGAA AATATCATCT TTGGTGTTTC CTATC

3 5

(2) INFORMATION FOR SEQ ID NO:322:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:322:

CCTTAAAGAA AATATCATCT TTGGTGTTTC CTAAA

3 5

(2) INFORMATION FOR SEQ ID NO:323:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:323:

CCTTAAAGAA AATAAATAA TTGGTGTTTC CTAAA

3 5

(2) INFORMATION FOR SEQ ID NO:324:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:324:

-continued

GGAAGTCTCC CATTTTAATT

20

(2) INFORMATION FOR SEQ ID NO:325:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:325:

CCTTCAGAGG GTAAAAATTAA

20

(2) INFORMATION FOR SEQ ID NO:326:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:326:

CCTTCAGAGK GTAAAAATTAA

20

(2) INFORMATION FOR SEQ ID NO:327:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:327:

CCTTCAGAGT GTAAAAATTAA

20

(2) INFORMATION FOR SEQ ID NO:328:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:328:

CCTTCAGAGG GTAAAAATCA

19

(2) INFORMATION FOR SEQ ID NO:329:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:329:

CCTTCAGAGG GTAAAAATTA

19

(2) INFORMATION FOR SEQ ID NO:330:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:330:

GATTTCAGAGT GTAAAAATAC

19

(2) INFORMATION FOR SEQ ID NO:331:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:331:

AAAAAAGAGT GTAAATGA

19

(2) INFORMATION FOR SEQ ID NO:332:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:332:

CATTAAAGAA AATAACATCA TTGGTGTTTC CTATG

35

(2) INFORMATION FOR SEQ ID NO:333:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 648 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:333:

AACAAACCTA CCCACCCCTA ACAGTACATA GTACATAAAG CCATTTACCG TACATAGCAC	60
ATTACAGTCA AATCCCTTCT CGTCCCCATG GATGACCCCC CTCAGATAGG GGTCCCTTGA	120
CCACCATCCT CCGTGAAATC AATATCCCGC ACAAGAGTGC TACTCTCCTC GCTCCGGGGC	180
CATAACACTT GGGGGTAGCT AAAGTGAAGT GTATCCGACA TCTGGTTCCT ACTTCAGGGT	240
CATAAAGCCT AAATAGCCCA CACGTTCCCC TTAATAAGA CATCACGATG GATCACAGGT	300
CTATCACCCCT ATTAACCACT CACGGGAGCT CTCCATGCAT TTGGTATTTT CGTCTGGGGG	360
GTATGCACGC GATAGCATTG CGAGACGCTG GAGCCGGAGC ACCCTATGTC GCAGTATCTG	420
TCTTTGATTG CTGCCTCATC CTATTATTTA TCGCACCTAC GTTCAATATT ACAGGGCGAAC	480
ATACTTACTA AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA ACAATTGAAT	540
GTCTGCACAG CCACITTCOA CACAGACATC ATAACAAAAA ATTTCCACCA AACCCCCCCT	600
CTCCCCCGCT TCTGGCCACA GCACCTAAAC ACATCTCTGC CAAACCCC	648

(2) INFORMATION FOR SEQ ID NO:334:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:334:

GATGCTGAGG AG

1 2

(2) INFORMATION FOR SEQ ID NO:335:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:335:

CTCCTCCCCG GT

1 2

(2) INFORMATION FOR SEQ ID NO:336:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:336:

ACTCCTCCCC GG

1 2

(2) INFORMATION FOR SEQ ID NO:337:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:337:

GACTCCTCCC CG

1 2

(2) INFORMATION FOR SEQ ID NO:338:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:338:

CGACTCCTCC CC

1 2

(2) INFORMATION FOR SEQ ID NO:339:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:339:

ACGACTCCTC CC

12

(2) INFORMATION FOR SEQ ID NO:340:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:340:

TACGACTCCT CC

12

(2) INFORMATION FOR SEQ ID NO:341:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:341:

CTACGACTCC TC

12

(2) INFORMATION FOR SEQ ID NO:342:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:342:

TCTACGACTC CT

12

(2) INFORMATION FOR SEQ ID NO:343:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:343:

TTCTACGACT CC

12

(2) INFORMATION FOR SEQ ID NO:344:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:344:

-continued

ATTCTACGAC TC

12

(2) INFORMATION FOR SEQ ID NO:345:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:345:

TATTCTACGA CT

12

(2) INFORMATION FOR SEQ ID NO:346:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:346:

CTATTCTACG AC

12

(2) INFORMATION FOR SEQ ID NO:347:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:347:

CCTATTCTAC GA

12

(2) INFORMATION FOR SEQ ID NO:348:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:348:

TCCTCCCCGG

10

(2) INFORMATION FOR SEQ ID NO:349:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:349:

CTCCTCCCCG

10

(2) INFORMATION FOR SEQ ID NO:350:

-continued

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:350:

ACTCCTCCCC

10

(2) INFORMATION FOR SEQ ID NO:351:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:351:

GACTCCTCCC

10

(2) INFORMATION FOR SEQ ID NO:352:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:352:

CGACTCCTCC

10

(2) INFORMATION FOR SEQ ID NO:353:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:353:

ACGACTCCTC

10

(2) INFORMATION FOR SEQ ID NO:354:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:354:

TACGACTCCT

10

(2) INFORMATION FOR SEQ ID NO:355:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-continued

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:355:

CTACGACTCC

10

(2) INFORMATION FOR SEQ ID NO:356:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:356:

TCTACGACTC

10

(2) INFORMATION FOR SEQ ID NO:357:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:357:

TTCTACGACT

10

(2) INFORMATION FOR SEQ ID NO:358:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:358:

ATTCTACGAC

10

(2) INFORMATION FOR SEQ ID NO:359:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (probe)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:359:

TATTCTACGA

10

(2) INFORMATION FOR SEQ ID NO:360:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 184 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: DNA (oligonucleotide)

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:360:

-continued

TACTCCCTG	CCCTCAACAA	GATGTTTTGC	CAACTGGCCA	AGACCTGCCC	TGTGCAGCWG	60
KGGGWWGATT	CCACACCCCC	GCCCGGCACC	CGCGTCCGCG	CCATGGCCAT	CTACAAGCAG	120
TCACAGCACA	TGACGGAGGW	WGKGAGGCGC	TGCCCCACC	ATGAGCGCYG	CYCAGATAGC	180
SAYG						184

We claim:

1. An array of oligonucleotide probes immobilized on a solid support, said array having at least 100 probes and no more than 100,000 different oligonucleotide probes 9 to 20 nucleotides in length occupying separate known sites in said array, said oligonucleotide probes comprising at least four sets of probes: (1) a first set that is exactly complementary to a reference sequence and comprises probes that completely span the reference sequence and, relative to the reference sequence, overlap one another in sequence; and (2) three additional sets of probes, each of which is identical to said first set of probes but for at least one different nucleotide, which different nucleotide is located in the same position in each of the three additional sets but which is a different nucleotide in each set.
2. The array of claim 1, further comprising a fourth additional set of probes, which fourth additional set is identical to probes in the first set.
3. The array of claim 1, wherein said reference sequence is a double-stranded nucleic acid and probes complementary to both strands of said reference are in said array.
4. The array of claim 1, wherein said probes are 12 to 17 nucleotides in length.
5. The array of claim 4, wherein said probes are 15 nucleotides in length and attached by a covalent linkage to a site on a 3'-end of said probes, and said different nucleotide is located at position 7, relative to the 3'-end of said probes.
6. The array of claim 1, wherein said reference sequence is exon 10 of a CFTR gene, and said array has between 1000 and 100,000 oligonucleotide probes 10 to 18 nucleotides in length.
7. The array of claim 6, wherein said array comprises a set of probes comprising a specific nucleotide sequence selected from the group of sequences consisting of:
 3'-TTTATAXTAG (SEQ ID. NO:302);
 3'-TTATAGXAGA (SEQ ID. NO:303);
 3'-TATAGTXGAA (SEQ ID. NO:304);
 3'-ATAGTAXAAA (SEQ ID. NO:305);
 3'-TAGTAGXAAC (SEQ ID. NO:306);
 3'-AGTAGAXACC (SEQ ID. NO:307);
 3'-GTAGAAXCCA (SEQ ID. NO:308);
 3'-TAGAAAXCAC (SEQ ID. NO:309); and
 3'-AGAAACXACA (SEQ ID. NO:310); wherein each set comprises 4 probes, and X is individually A, G, C, and T for each set.
8. The array of claim 6, wherein said group of sequences consists of:
 3'-TTTATAXTAGAAACC (SEQ ID. NO:9);
 3'-TTATAGXAGAAACCA (SEQ ID. NO:10);
 3'-TATAGTXGAAACCAC (SEQ ID. NO:11);
 3'-ATAGTAXAAACCACA (SEQ ID. NO:12);
10. 3'-TAGTAGXAACCACAA (SEQ ID. NO:13);
 3'-AGTAGAXACCACAAA (SEQ ID. NO:14);
 3'-GTAGAAXCCACAAAG (SEQ ID. NO:15);
 3'-TAGAAAXCACAAAGG (SEQ ID. NO:16); and
 15. 3'-AGAAACXACAAAGGA (SEQ ID. NO:17); wherein each set comprises 4 probes, and X is individually A, G, C, and T for each set.
9. The array of claim 1, wherein said reference sequence is a sequence of a D-loop region of human mitochondrial DNA.
10. The array of claim 9, wherein said probes are 15 nucleotides in length, and said array comprises a first set of probes exactly complementary to a sequence contained in a sequence bounded by positions 16280 to 356 of the reference sequence and four additional sets of probes identical to said first set but for position 7, relative to a 3'-end of a probe, which 3'-end is covalently attached to the substrate, where, for each of the four additional probe sets, a different nucleotide is located, such that, for each probe in said first set, there is an identical probe in one of the four additional sets, and such that the array has between 2500 and 100,000 oligonucleotide probes.
11. The array of claim 1, wherein said reference sequence is a sequence from an exon of a human p53 gene.
12. The array of claim 11, wherein said reference sequence comprises at least a 60 nucleotide contiguous sequence from exon 6 of a p53 gene.
13. The array of claim 11, wherein said reference sequence is exon 5 of a p53 gene, said probes are 17 nucleotides long, and said array comprises a first set of probes exactly complementary to said sequence and at least three additional sets of probes, each set comprising probes identical to said first set but for a nucleotide at position 7, relative to a 3'-end of a probe, which 3'-end is covalently attached to the substrate, which nucleotide is different from a nucleotide at this position in a corresponding probe of said first set.
14. The array of claim 1, wherein said probes are oligodeoxynucleotides.
15. The array of claim 1, wherein said array has between 10,000 and 100,000 probes.
16. The array of claim 1, wherein the reference sequence is from a human immunodeficiency virus.
17. The array of claim 16, wherein the reference sequence is from a reverse transcriptase gene of the human immunodeficiency virus.
18. The array of claim 1, wherein said probes are immobilized to said solid support via a linker.

* * * * *

I

This Page Blank (uspto)



US006156501A

United States Patent [19]

McGall et al.

[11] **Patent Number:** 6,156,501[45] **Date of Patent:** *Dec. 5, 2000[54] **ARRAYS OF MODIFIED NUCLEIC ACID PROBES AND METHODS OF USE**

[75] **Inventors:** Glenn Hugh McGall; Charles Garrett Mlyada, both of Mountain View; Maureen T. Cronin, Los Altos; Jennifer Dee Tan, Newark; Mark S. Chee, Palo Alto, all of Calif.

[73] **Assignee:** Affymetrix, Inc., Santa Clara, Calif.

[*] **Notice:** This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] **Appl. No.:** 08/630,427

[22] **Filed:** Apr. 3, 1996

Related U.S. Application Data

[63] Continuation-in-part of application No. 08/440,742, May 10, 1995, abandoned, which is a continuation-in-part of application No. PCT/US94/12305, Oct. 26, 1994, which is a continuation-in-part of application No. 08/284,064, Aug. 2, 1994, abandoned, which is a continuation-in-part of application No. 08/143,312, Oct. 26, 1993, abandoned.

[51] **Int. Cl.⁷** C12Q 1/68; C07H 21/00

[52] **U.S. Cl.** 435/6; 422/50; 422/68.1; 435/283.1; 435/287.1; 435/287.2; 435/288.3; 435/288.7; 435/289.1; 435/299.1; 435/305.1; 436/501; 536/22.1; 536/24.1; 536/24.3; 536/24.31; 536/24.32; 536/24.33

[58] **Field of Search** 422/50, 68.1; 435/6, 435/810, 283.1, 287.1, 287.2, 288.3, 288.7, 289.1, 299.1, 305.1; 436/501; 536/23.1, 24.1, 22.1, 24.3-24.33; 935/77, 78

[56] **References Cited****U.S. PATENT DOCUMENTS**

5,002,867 3/1991 Macevitz 435/6

5,143,854 9/1992 Pirrung et al. 436/518
5,200,051 4/1993 Cozzette et al. 204/403
5,202,231 4/1993 Drmanac et al. 435/6
5,217,866 6/1993 Summerton et al. 435/6
5,412,087 5/1995 McGall et al. 536/24.3
5,474,796 12/1995 Brennan 427/2.13
5,484,908 1/1996 Froehler et al. 536/24.31
5,527,681 6/1996 Holmes 435/6
5,556,752 9/1996 Lockhart et al. 435/6
5,556,961 9/1996 Foote et al. 536/27.1
5,604,097 2/1997 Brenner 435/6
5,800,992 9/1998 Fodor et al. 435/6
5,821,060 10/1998 Arlinghaus et al. 435/6
B1 4,683,202 11/1990 Mullis 435/91

FOREIGN PATENT DOCUMENTS

8605518 9/1986 WIPO .
WO 89/10977 11/1989 WIPO .
WO 89/11548 11/1989 WIPO .
WO90/04652 5/1990 WIPO C12Q 1/68
WO 92/10092 6/1992 WIPO .
WO 93/17126 9/1993 WIPO .

OTHER PUBLICATIONS

Durland, Ross H., et al. (1995) "Selective Binding of Pyrido[2, 3-d]pyrimidine 2'-Deoxyribonucleoside to AT Base Pairs in Antiparallel Triple Helices", *Bioconjugate Chem* 6:278-282.

Primary Examiner—Ardin H. Marschel

Attorney, Agent, or Firm—Townsend and Townsend and Crew

[57] **ABSTRACT**

Oligonucleotide analogue arrays attached to solid substrates and methods related to the use thereof are provided. The oligonucleotide analogues hybridize to nucleic acids with either higher or lower specificity than corresponding unmodified oligonucleotides. Target nucleic acids which comprise nucleotide analogues are bound to oligonucleotide and oligonucleotide analogue arrays.

72 Claims, 5 Drawing Sheets

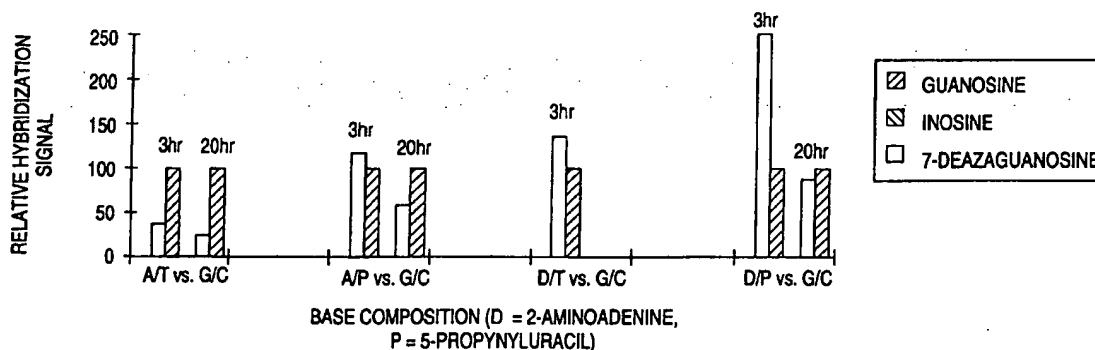


FIG. 1A

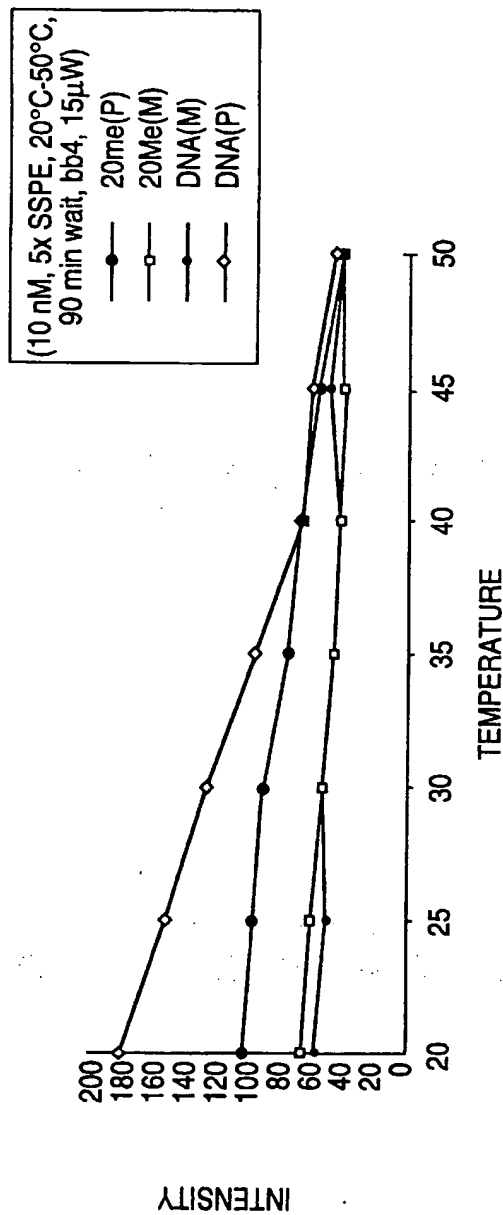
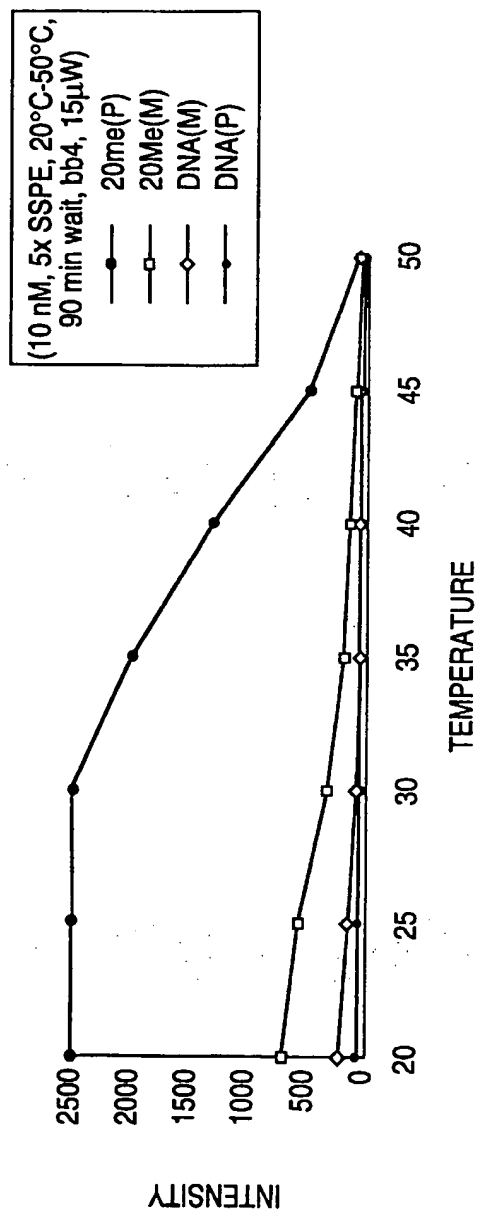


FIG. 1B



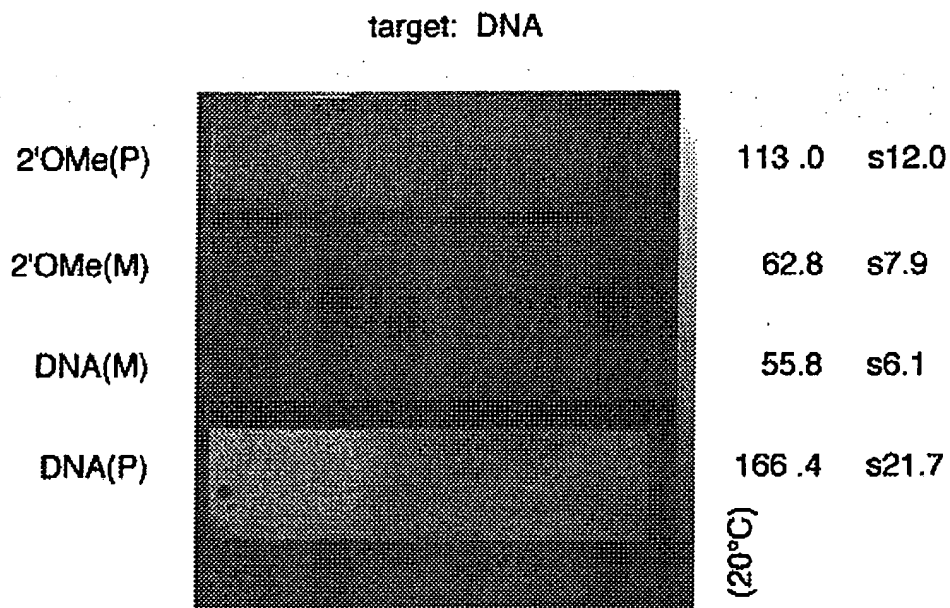


FIG. 1C

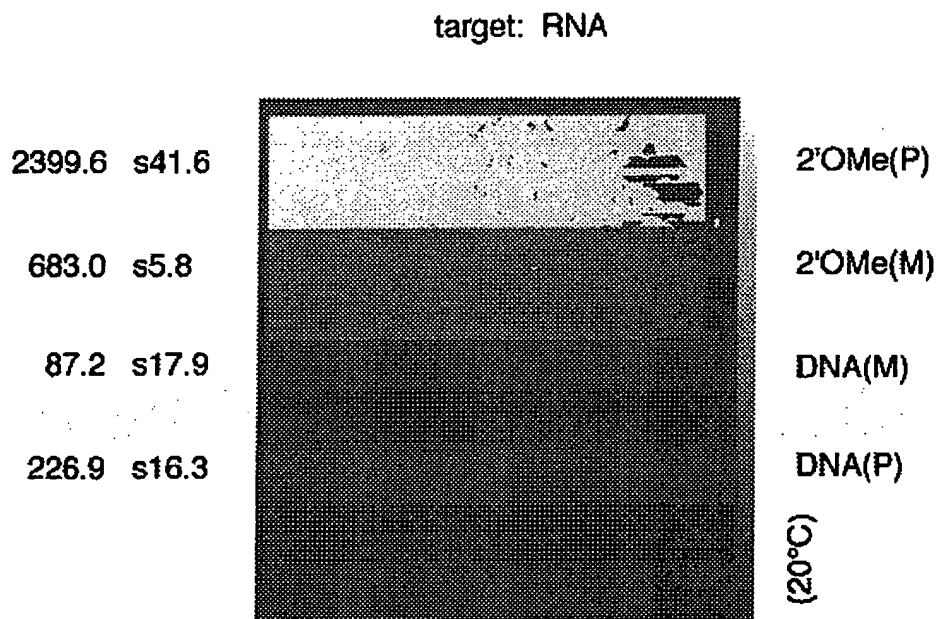


FIG. 1D

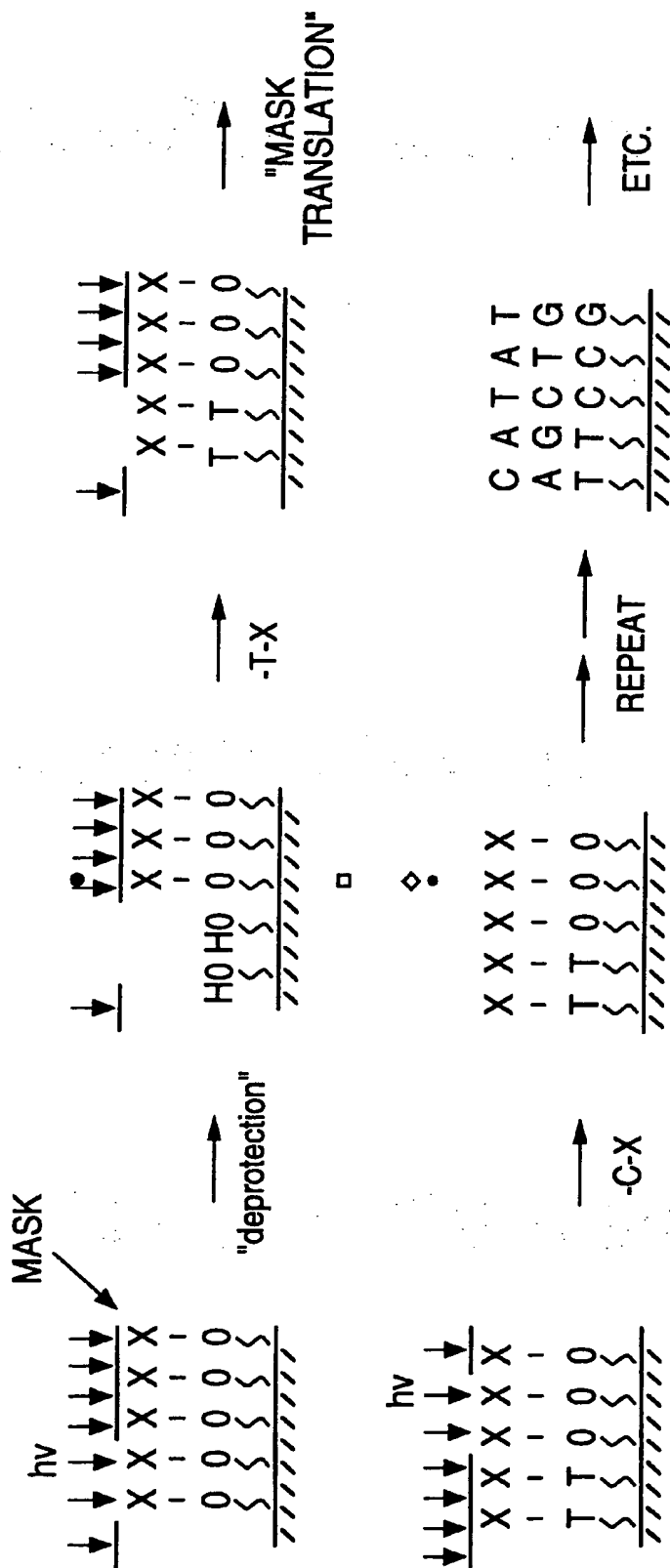
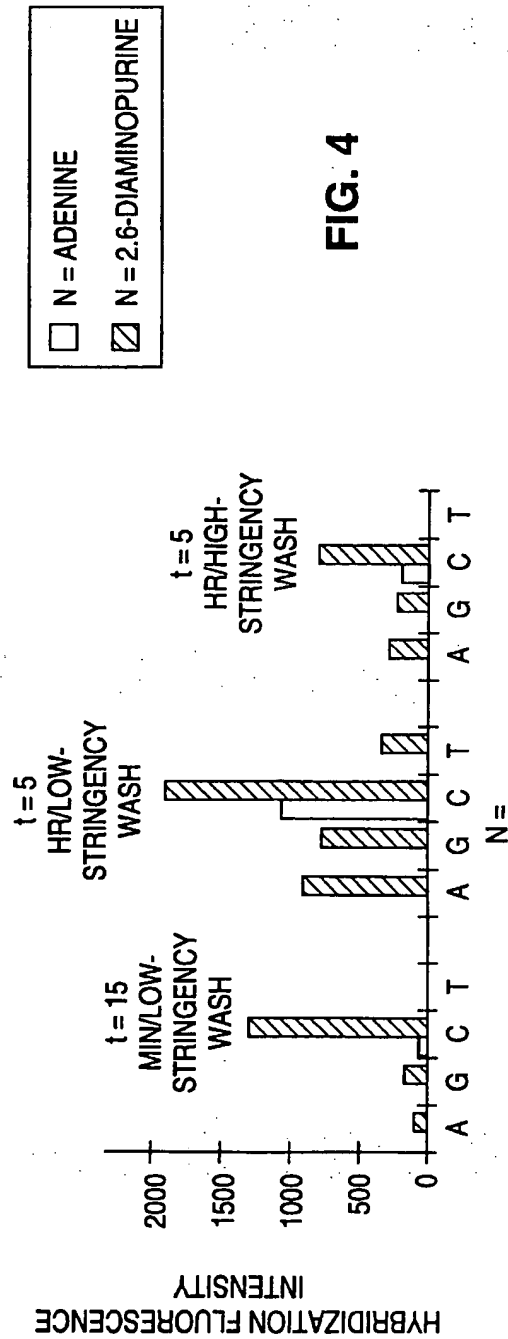
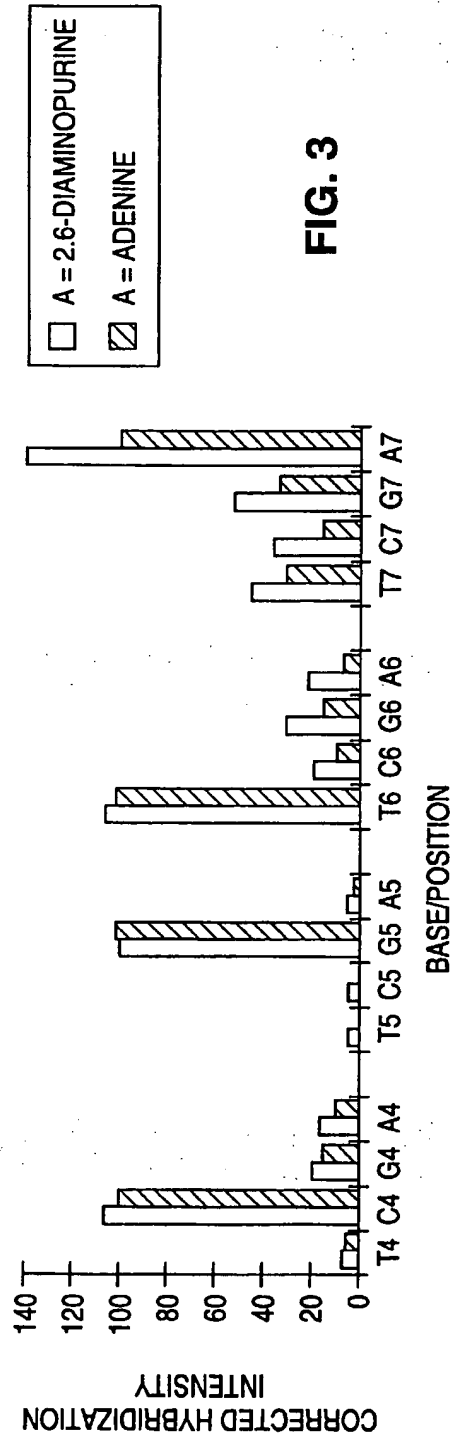
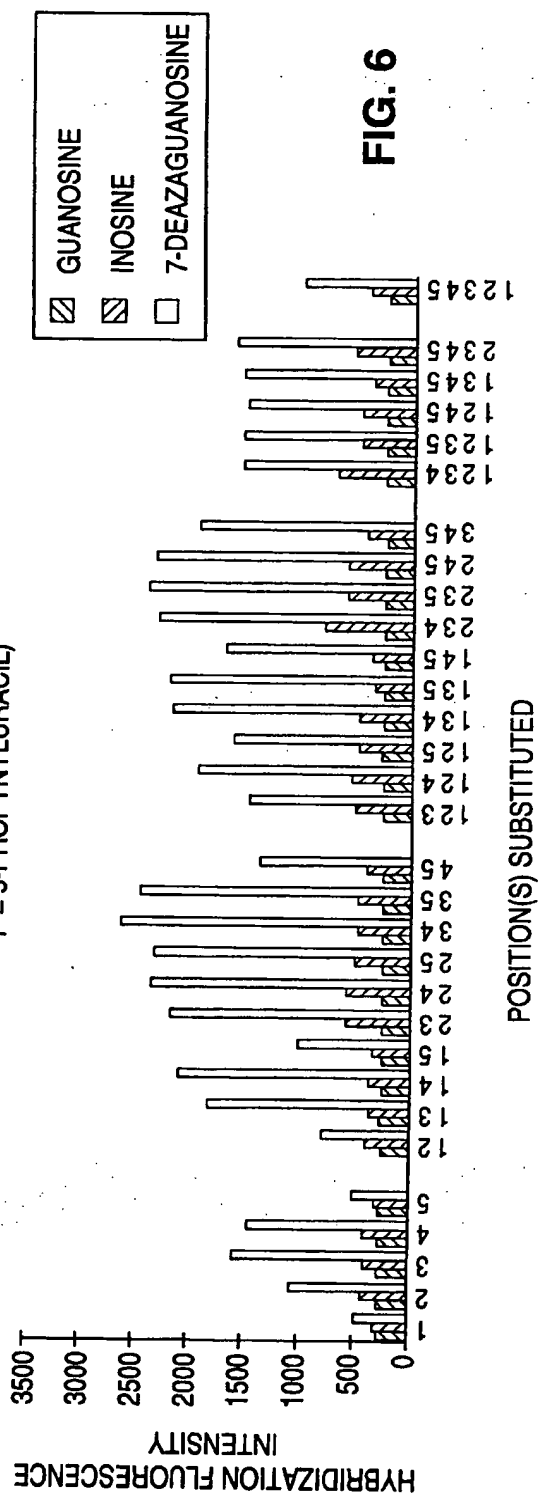
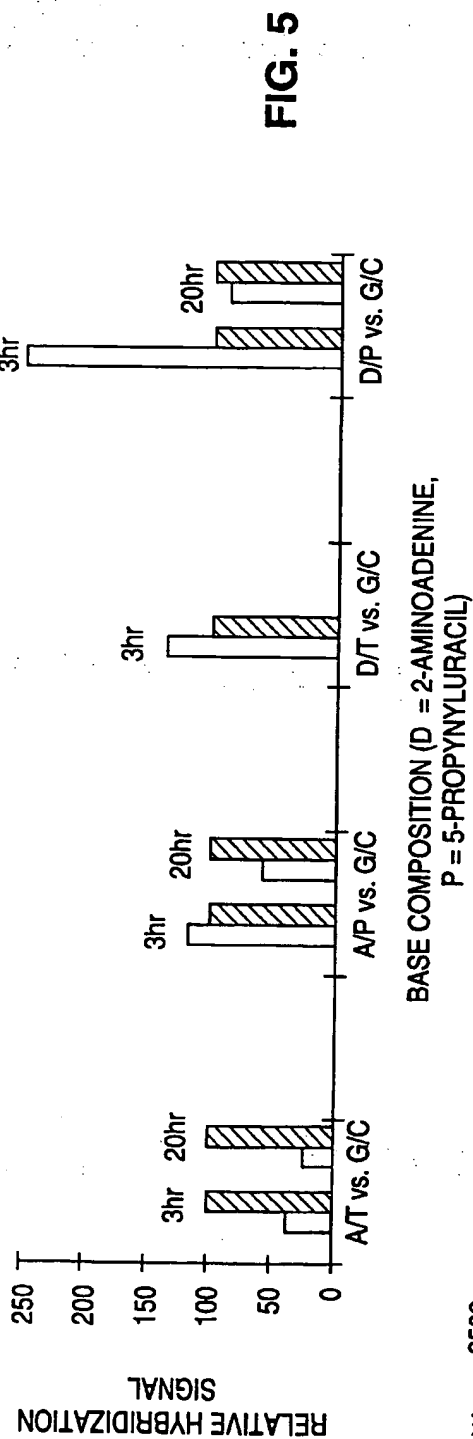


FIG. 2





ARRAYS OF MODIFIED NUCLEIC ACID PROBES AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. Ser. No. 08/440,742 filed May 10, 1995 abandoned, which is a continuation-in-part of PCT application (designating the United States) SN PCT/US94/12305 filed Oct. 26, 1994, which is a continuation-in-part of U.S. Ser. No. 08/284,064 filed Aug. 2, 1994 abandoned, which is a continuation-in-part of U.S. Ser. No. 08/143,312 filed Oct. 26, 1993 abandoned, each of which is incorporated herein by reference in its entirety for all purposes.

FIELD OF THE INVENTION

The present invention provides probes comprised of nucleotide analogues immobilized in arrays on solid substrates for analyzing molecular interactions of biological interest, and target nucleic acids comprised of nucleotide analogues. The invention therefore relates to the molecular interaction of polymers immobilized on solid substrates including related chemistry, biology, and medical diagnostic uses.

BACKGROUND OF THE INVENTION

The development of very large scale immobilized polymer synthesis (VLSIPS™) technology provides pioneering methods for arranging large numbers of oligonucleotide probes in very small arrays. See, U.S. application Ser. No. 07/805,727 now U.S. Pat. No. 5,424,186 and PCT patent publication Nos. WO 90/15070 and 92/10092, each of which is incorporated herein by reference for all purposes. U.S. patent application Ser. No. 08/082,937, filed Jun. 25, 1993, and incorporated herein for all purposes, describes methods for making arrays of oligonucleotide probes that are used, e.g., to determine the complete sequence of a target nucleic acid and/or to detect the presence of a nucleic acid with a specified sequence.

VLSIPS™ technology provides an efficient means for large scale production of miniaturized oligonucleotide arrays for sequencing by hybridization (SBH), diagnostic testing for inherited or somatically acquired genetic diseases, and forensic analysis. Other applications include determination of sequence specificity of nucleic acids, protein-nucleic acid complexes and other polymer-polymer interactions.

SUMMARY OF THE INVENTION

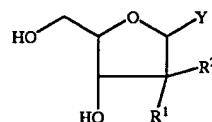
The present invention provides arrays of oligonucleotide analogues attached to solid substrates. Oligonucleotide analogues have different hybridization properties than oligonucleotides based upon naturally occurring nucleotides. By incorporating oligonucleotide analogues into the arrays of the invention, hybridization to a target nucleic acid is optimized.

The oligonucleotide analogue arrays have virtually any number of different members, determined largely by the number or variety of compounds to be screened against the array in a given application. In one group of embodiments, the array has from 10 up to 100 oligonucleotide analogue members. In other groups of embodiments, the arrays have between 100 and 10,000 members, and in yet other embodiments the arrays have between 10,000 and 1,000,000 members. In preferred embodiments, the array will have a

density of more than 100 members at known locations per cm², or more preferably, more than 1000 members per cm². In some embodiments, the arrays have a density of more than 10,000 members per cm².

The solid substrate upon which the array is constructed includes any material upon which oligonucleotide analogues are attached in a defined relationship to one another, such as beads, arrays, and slides. Especially preferred oligonucleotide analogues of the array are between about 5 and about 20 nucleotides, nucleotide analogues or a mixture thereof in length.

In one group of embodiments, nucleoside analogues incorporated into the oligonucleotide analogues of the array will have the chemical formula:



wherein R¹ and R² are independently selected from the group consisting of hydrogen, methyl, hydroxy, alkoxy (e.g., methoxy, ethoxy, propoxy, allyloxy, and propargyloxy), alkylthio, halogen (Fluorine, Chlorine, and Bromine), cyano, and azido, and wherein Y is a heterocyclic moiety, e.g., a base selected from the group consisting of purines, purine analogues, pyrimidines, pyrimidine analogues, universal bases (e.g., 5-nitroindole) or other groups or ring systems capable of forming one or more hydrogen bonds with corresponding moieties on alternate strands within a double- or triple-stranded nucleic acid or nucleic acid analogue, or other groups or ring systems capable of forming nearest-neighbor base-stacking interactions within a double- or triple-stranded complex. In other embodiments, the oligonucleotide analogues are not constructed from nucleosides, but are capable of binding to nucleic acids in solution due to structural similarities between the oligonucleotide analogue and a naturally occurring nucleic acid. An example of such an oligonucleotide analogue is a peptide nucleic acid or polyamide nucleic acid in which bases which hydrogen bond to a nucleic acid are attached to a polyamide backbone.

The present invention also provides target nucleic acids hybridized to oligonucleotide arrays. In the target nucleic acids of the invention, nucleotide analogues are incorporated into the target nucleic acid, altering the hybridization properties of the target nucleic acid to an array of oligonucleotide probes. Typically, the oligonucleotide probe arrays also comprise nucleotide analogues.

The target nucleic acids are typically synthesized by providing a nucleotide analogue as a reagent during the enzymatic copying of a nucleic acid. For instance, nucleotide analogues are incorporated into polynucleic acid analogues using taq polymerase in a PCR reaction. Thus, a nucleic acid containing a sequence to be analyzed is typically amplified in a PCR or RNA amplification procedure with nucleotide analogues, and the resulting target nucleic acid analogue amplicon is hybridized to a nucleic acid analogue array.

Oligonucleotide analogue arrays and target nucleic acids are optionally composed of oligonucleotide analogues which are resistant to hydrolysis or degradation by nuclease enzymes such as RNAase A. This has the advantage of providing the array or target nucleic acid with greater longevity by rendering it resistant to enzymatic degradation.

For example, analogues comprising 2'-O-methyloligoribonucleotides are resistant to RNAase A.

Oligonucleotide analogue arrays are optionally arranged into libraries for screening compounds for desired characteristics, such as the ability to bind a specified oligonucleotide analogue, or oligonucleotide analogue-containing structure. The libraries also include oligonucleotide analogue members which form conformationally-restricted probes, such as unimolecular double-stranded probes or unimolecular double-stranded probes which present a third chemical structure of interest. For instance, the array of oligonucleotide analogues optionally include a plurality of different members, each member having the formula: $Y-L^1-X^1-L^2-X^2$, wherein Y is a solid substrate, X^1 and X^2 are complementary oligonucleotides containing at least one nucleotide analogue, L^1 is a spacer, and L^2 is a linking group having sufficient length such that X^1 and X^2 form a double-stranded oligonucleotide. An array of such members comprise a library of unimolecular double-stranded oligonucleotide analogues. In another embodiment, the members of the array of oligonucleotide are arranged to present a moiety of interest within the oligonucleotide analogue probes of the array. For instance, the arrays are optionally conformationally restricted, having the formula $-X^{11}-Z-X^{12}$, wherein X^{11} and X^{12} are complementary oligonucleotides or oligonucleotide analogues and Z is a chemical structure comprising the binding site of interest.

Oligonucleotide analogue arrays are synthesized on a solid substrate by a variety of methods, including light-directed chemical coupling, and selectively flowing synthetic reagents over portions of the solid substrate. The solid substrate is prepared for synthesis or attachment of oligonucleotides by treatment with suitable reagents. For example, glass is prepared by treatment with silane reagents.

The present invention provides methods for determining whether a molecule of interest binds members of the oligonucleotide analogue array. For instance, in one embodiment, a target molecule is hybridized to the array and the resulting hybridization pattern is determined. The target molecule includes genomic DNA, cDNA, unspliced RNA, mRNA, and rRNA, nucleic acid analogues, proteins and chemical polymers. The target molecules are optionally amplified prior to being hybridized to the array, e.g., by PCR, LCR, or cloning methods.

The oligonucleotide analogue members of the array used in the above methods are synthesized by any described method for creating arrays. In one embodiment, the oligonucleotide analogue members are attached to the solid substrate, or synthesized on the solid substrate by light-directed very large scale immobilized polymer synthesis, e.g., using photo-removable protecting groups during synthesis. In another embodiment, the oligonucleotide members are attached to the solid substrate by forming a plurality of channels adjacent to the surface of said substrate, placing selected monomers in said channels to synthesize oligonucleotide analogues at predetermined portions of selected regions, wherein the portion of the selected regions comprise oligonucleotide analogues different from oligonucleotide analogues in at least one other of the selected regions, and repeating the steps with the channels formed along a second portion of the selected regions. The solid substrate is any suitable material as described above, including beads, slides, and arrays, each of which is constructed from, e.g., silica, polymers and glass.

DEFINITIONS

An "Oligonucleotide" is a nucleic acid sequence composed of two or more nucleotides. An oligonucleotide is

optionally derived from natural sources, but is often synthesized chemically. It is of any size. An "oligonucleotide analogue" refers to a polymer with two or more monomeric subunits, wherein the subunits have some structural features in common with a naturally occurring oligonucleotide which allow it to hybridize with a naturally occurring oligonucleotide in solution. For instance, structural groups are optionally added to the ribose or base of a nucleoside for incorporation into an oligonucleotide, such as a methyl or allyl group at the 2'-O position on the ribose, or a fluoro group which substitutes for the 2'-O group, or a bromo group on the ribonucleoside base. The phosphodiester linkage, or "sugar-phosphate backbone" of the oligonucleotide analogue is substituted or modified, for instance with methyl phosphonates or O-methyl phosphates. Another example of an oligonucleotide analogue for purposes of this disclosure includes "peptide nucleic acids" in which native or modified nucleic acid bases are attached to a polyamide backbone. Oligonucleotide analogues optionally comprise a mixture of naturally occurring nucleotides and nucleotide analogues. However, an oligonucleotide which is made entirely of naturally occurring nucleotides (i.e., those comprising DNA or RNA), with the exception of a protecting group on the end of the oligonucleotide, such as a protecting group used during standard nucleic acid synthesis is not considered an oligonucleotide analogue for purposes of this invention.

A "nucleoside" is a pentose glycoside in which the aglycone is a heterocyclic base; upon the addition of a phosphate group the compound becomes a nucleotide. The major biological nucleosides are β -glycoside derivatives of D-ribose or D-2-deoxyribose. Nucleotides are phosphate esters of nucleosides which are generally acidic in solution due to the hydroxy groups on the phosphate. The nucleosides of DNA and RNA are connected together via phosphate units attached to the 3' position of one pentose and the 5' position of the next pentose. Nucleotide analogues and/or nucleoside analogues are molecules with structural similarities to the naturally occurring nucleotides or nucleosides as discussed above in the context of oligonucleotide analogues.

A "nucleic acid reagent" utilized in standard automated oligonucleotide synthesis typically carries a protected phosphate on the 3' hydroxyl of the ribose. Thus, nucleic acid reagents are referred to as nucleotides, nucleotide reagents, nucleoside reagents, nucleoside phosphates, nucleoside-3'-phosphates, nucleoside phosphoramidites, phosphoramidites, nucleoside phosphonates, phosphonates and the like. It is generally understood that nucleotide reagents carry a reactive, or activatable, phosphoryl or phosphonyl moiety in order to form a phosphodiester linkage.

A "protecting group" as used herein, refers to any of the groups which are designed to block one reactive site in a molecule while a chemical reaction is carried out at another reactive site. More particularly, the protecting groups used herein are optionally any of those groups described in Greene, et al., *Protective Groups In Organic Chemistry*, 2nd Ed., John Wiley & Sons, New York, NY, 1991, which is incorporated herein by reference. The proper selection of protecting groups for a particular synthesis is governed by the overall methods employed in the synthesis. For example, in "light-directed" synthesis, discussed herein, the protecting groups are photolabile protecting groups such as NVOC, MeNPoc, and those disclosed in co-pending Application PCT/US93/10162 (filed Oct. 22, 1993), incorporated herein by reference. In other methods, protecting groups are removed by chemical methods and include groups such as FMOC, DMT and others known to those of skill in the art.

A "purine" is a generic term based upon the specific compound "purine" having a skeletal structure derived from the fusion of a pyrimidine ring and an imidazole ring. It is generally, and herein, used to describe a generic class of compounds which have an atom or a group of atoms added to the parent purine compound, such as the bases found in the naturally occurring nucleic acids adenine (6-aminopurine) and guanine (2-amino-6-oxopurine), or less commonly occurring molecules such as 2-amino-adenine, N⁶-methyladenine, or 2-methylguanine.

A "purine analogue" has a heterocyclic ring with structural similarities to a purine, in which an atom or group of atoms is substituted for an atom in the purine ring. For instance, in one embodiment, one or more N atoms of the purine heterocyclic ring are replaced by C atoms.

A "pyrimidine" is a compound with a specific heterocyclic diazine ring structure, but is used generically by persons of skill and herein to refer to any compound having a 1,3-diazine ring with minor additions, such as the common nucleic acid bases cytosine, thymine, uracil, 5-methylcytosine and 5-hydroxymethylcytosine, or the non-naturally occurring 5-bromo-uracil.

A "pyrimidine analogue" is a compound with structural similarity to a pyrimidine, in which one or more atom in the pyrimidine ring is substituted. For instance, in one embodiment, one or more of the N atoms of the ring are substituted with C atoms.

A "solid substrate" has fixed organizational support matrix, such as silica, polymeric materials, or glass. In some embodiments, at least one surface of the substrate is partially planar. In other embodiments it is desirable to physically separate regions of the substrate to delineate synthetic regions, for example with trenches, grooves, wells or the like. Example of solid substrates include slides, beads and arrays.

DESCRIPTION OF THE DRAWINGS

FIG. 1 shows four panels (FIG. 1A, FIG. 1B, FIG. 1C and FIG. 1D). FIGS. 1A and 1B graphically display the difference in fluorescence intensity between the matched and mismatched DNA probes. FIGS. 1C and 1D illustrate the difference in fluorescence intensity verses location on an example chip for DNA and RNA targets, respectively.

FIG. 2 is a graphic illustration of specific light-directed chemical coupling of oligonucleotide analogue monomers to an array.

FIG. 3 shows the relative efficiency and specificity of hybridization for immobilized probe arrays containing adenine versus probe arrays containing 2,6-diaminopurine nucleotides. (3'-CATCGTAGAA-5' (SEQ ID NO:1)).

FIG. 4 shows the effect of substituting adenine with 2,6-diaminopurine (D) in immobilized poly-dA probe arrays. (AAAAANAAAAA (SEQ ID NO:2)).

FIG. 5 shows the effects of substituting 5-propynyl-2'-deoxyuridine and 2-amino-2'-deoxyadenosine in AT arrays on hybridization to a target nucleic acid. (ATATAATATA (SEQ ID NO:3) and CGCGCCGCGC (SEQ ID NO:4)).

FIG. 6 shows the effects of dI and 7-deaza-dG substitutions in oligonucleotide arrays. (3'-ATGTT(G1G2G3G4G5)CGGGT-5' (SEQ ID NO:5)).

DETAILED DESCRIPTION

Methods of synthesizing desired single stranded oligonucleotide and oligonucleotide analogue sequences are known to those of skill in the art. In particular, methods of

synthesizing oligonucleotides and oligonucleotide analogues are found in, for example, *Oligonucleotide Synthesis: A Practical Approach*, Gait, ed., IRL Press, Oxford (1984); W. H. A. Kuipers *Nucleic Acids Research* 18(17), 5197 (1994); K. L. Dueholm *J. Org. Chem.* 59, 5767-5773 (1994), and S. Agrawal (ed.) *Methods in Molecular Biology*, volume 20, each of which is incorporated herein by reference in its entirety for all purposes. Synthesizing unimolecular double-stranded DNA in solution has also been described. See, copending application Ser. No. 08/327,687, now U.S. Pat. No. 5,556,752 which is incorporated herein for all purposes.

Improved methods of forming large arrays of oligonucleotides, peptides and other polymer sequences with a minimal number of synthetic steps are known. See, Pirrung et al., U.S. Pat. No. 5,143,854 (see also, PCT Application No. WO 90/15070) and Fodor et al., PCT Publication No. WO 92/10092, which are incorporated herein by reference, which disclose methods of forming vast arrays of peptides, oligonucleotides and other molecules using, for example, light-directed synthesis techniques. See also, Fodor et al., (1991) *Science*, 251, 767-77 which is incorporated herein by reference for all purposes. These procedures for synthesis of polymer arrays are now referred to as VLSIPS™ procedures.

Using the VLSIPS™ approach, one heterogenous array of polymers is converted, through simultaneous coupling at a number of reaction sites, into a different heterogenous array. See, U.S. application Ser. No. 07/796,243 now U.S. Pat. No. 5,384,261 and U.S. application Ser. No. 07/980,523 now U.S. Pat. No. 5,677,195, the disclosures of which are incorporated herein for all purposes.

The development of VLSIPS™ technology as described in the above-noted U.S. Pat. No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092 is considered pioneering technology in the fields of combinatorial synthesis and screening of combinatorial libraries. More recently, patent application Ser. No. 08/082,937, filed Jun. 25, 1993 (incorporated herein by reference), describes methods for making arrays of oligonucleotide probes that are used to check or determine a partial or complete sequence of a target nucleic acid and to detect the presence of a nucleic acid containing a specific oligonucleotide sequence.

Combinatorial Synthesis of Oligonucleotide Arrays

VLSIPS™ technology provides for the combinatorial synthesis of oligonucleotide arrays. The combinatorial VLSIPS™ strategy allows for the synthesis of arrays containing a large number of related probes using a minimal number of synthetic steps. For instance, it is possible to synthesize and attach all possible DNA 8mer oligonucleotides (4⁸, or 65,536 possible combinations) using only 32 chemical synthetic steps. In general, VLSIPS™ procedures provide a method of producing 4ⁿ different oligonucleotide probes on an array using only 4n synthetic steps.

In brief, the light-directed combinatorial synthesis of oligonucleotide arrays on a glass surface proceeds using automated phosphoramidite chemistry and chip masking techniques. In one specific implementation, a glass surface is derivatized with a silane reagent containing a functional group, e.g., a hydroxyl or amine group blocked by a photolabile protecting group. Photolysis through a photolithographic mask is used selectively to expose functional groups which are then ready to react with incoming 5'-photoprotected nucleoside phosphoramidites. See, FIG. 2. The phosphoramidites react only with those sites which are illuminated (and thus exposed by removal of the photolabile

blocking group). Thus, the phosphoramidites only add to those areas selectively exposed from the preceding step. These steps are repeated until the desired array of sequences have been synthesized on the solid surface. Combinatorial synthesis of different oligonucleotide analogues at different locations on the array is determined by the pattern of illumination during synthesis and the order of addition of coupling reagents.

In the event that an oligonucleotide analogue with a polyamide backbone is used in the VLSIPS™ procedure, it is generally inappropriate to use phosphoramidite chemistry to perform the synthetic steps, since the monomers do not attach to one another via a phosphate linkage. Instead, peptide synthetic method are substituted. See, e.g., Pirrung et al. U.S. Pat. No. 5,143,854.

Peptide nucleic acids are commercially available from, e.g., Biosearch, Inc. (Bedford, Mass.) which comprise a polyamide backbone and the bases found in naturally occurring nucleosides. Peptide nucleic acids are capable of binding to nucleic acids with high specificity, and are considered "oligonucleotide analogues" for purposes of this disclosure. Note that peptide nucleic acids optionally comprise bases other than those which are naturally occurring.

Hybridization of Nucleotide Analogues

The stability of duplexes formed between RNAs or DNAs are generally in the order of RNA:RNA>RNA:DNA>DNA:DNA, in solution. Long probes have better duplex stability with a target, but poorer mismatch discrimination than shorter probes (mismatch discrimination refers to the measured hybridization signal ratio between a perfect match probe and a single base mismatch probe. Shorter probes (e.g., 8-mers) discriminate mismatches very well, but the overall duplex stability is low. In order to optimize mismatch discrimination and duplex stability, the present invention provides a variety of nucleotide analogues incorporated into polymers and attached in an array to a solid substrate.

Altering the thermal stability (T_m) of the duplex formed between the target and the probe using, e.g., known oligonucleotide analogues allows for optimization of duplex stability and mismatch discrimination. One useful aspect of altering the T_m arises from the fact that Adenine-Thymine (A-T) duplexes have a lower T_m than Guanine-Cytosine (G-C) duplexes, due in part to the fact that the A-T duplexes have 2 hydrogen bonds per base-pair, while the G-C duplexes have 3 hydrogen bonds per base pair. In heterogeneous oligonucleotide arrays in which there is a non-uniform distribution of bases, it can be difficult to optimize hybridization conditions for all probes simultaneously. Thus, in some embodiments, it is desirable to destabilize G-C-rich duplexes and/or to increase the stability of A-T-rich duplexes while maintaining the sequence specificity of hybridization. This is accomplished, e.g., by replacing one or more of the native nucleotides in the probe (or the target) with certain modified, non-standard nucleotides. Substitution of guanine residues with 7-deazaguanine, for example, will generally destabilize duplexes, whereas substituting adenine residues with 2,6-diaminopurine will enhance duplex stability. A variety of other modified bases are also incorporated into nucleic acids to enhance or decrease overall duplex stability while maintaining specificity of hybridization. The incorporation of 6-aza-pyrimidine analogs into oligonucleotide probes generally decreases their binding affinity for complementary nucleic acids. Many 5-substituted pyrimidines substantially increase the stability of hybrids in which they have been substituted in place of the native pyrimidines in the

sequence. Examples include 5-bromo-, 5-methyl-, 5-propynyl-, 5-(imidazol-2-yl)- and 5-(thiazol-2-yl)-derivatives of cytosine and uracil.

Many modified nucleosides, nucleotides and various bases suitable for incorporation into nucleosides are commercially available from a variety of manufacturers, including the SIGMA chemical company (Saint Louis, Mo.), R&D systems (Minneapolis, Minn.), Pharmacia LKB Biotechnology (Piscataway, N.J.), CLONTECH Laboratories, Inc. (Palo Alto, Calif.), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, Wis.), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, Md.), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, Calif., and Applied Biosystems (Foster City, Calif.), as well as many other commercial sources known to one of skill. Methods of attaching bases to sugar moieties to form nucleosides are known. See, e.g., Lukevics and Zablocka (1991), *Nucleoside Synthesis: Organosilicon Methods* Ellis Horwood Limited Chichester, West Sussex, England and the references therein. Methods of phosphorylating nucleosides to form nucleotides, and of incorporating nucleotides into oligonucleotides are also known. See, e.g., Agrawal (ed) (1993) *Protocols for Oligonucleotides and Analogues, Synthesis and Properties*, Methods in Molecular Biology volume 20, Humana Press, Towota, N.J., and the references therein. See also, Crooke and Lebleu, and Sanghvi and Cook, and the references cited therein, both supra.

Groups are also linked to various positions on the nucleoside sugar ring or on the purine or pyrimidine rings which may stabilize the duplex by electrostatic interactions with the negatively charged phosphate backbone, or through hydrogen bonding interactions in the major and minor grooves. For example, adenosine and guanosine nucleotides are optionally substituted at the N² position with an imidazolyl propyl group, increasing duplex stability. Universal base analogues such as 3-nitropyrrole and 5-nitroindole are optionally included in oligonucleotide probes to improve duplex stability through base stacking interactions.

Selecting the length of oligonucleotide probes is also an important consideration when optimizing hybridization specificity. In general, shorter probe sequences are more specific than longer ones, in that the occurrence of a single-base mismatch has a greater destabilizing effect on the hybrid duplex. However, as the overall thermodynamic stability of hybrids decreases with length, in some embodiments it is desirable to enhance duplex stability for short probes globally. Certain modifications of the sugar moiety in oligonucleotides provide useful stabilization, and these can be used to increase the affinity of probes for complementary nucleic acid sequences. For example, 2'-O-methyl-, 2'-O-propyl-, and 2'-O-allyl-oligoribonucleotides have higher binding affinities for complementary RNA sequences than their unmodified counterparts. Probes comprised of 2'-fluoro-2'-deoxyoligoribonucleotides also form more stable hybrids with RNA than do their unmodified counterparts.

Replacement or substitution of the internucleotide phosphodiester linkage in oligo- or poly-nucleotides is also used to either increase or decrease the affinity of probe-target interactions. For example, substituting phosphodiester linkages with phosphorothioate or phosphorodithioate linkages generally lowers duplex stability, without affecting sequence specificity. Substitutions with a non-ionic methylphosphonate linkage (racemic, or preferably, Rp stereochemistry) have a stabilizing influence on hybrid formation. Neutral or cationic phosphoramidate linkages also result in enhanced

duplex stabilization. The phosphate diester backbone has been replaced with a variety of other stabilizing, non-natural linkages which have been studied as potential antisense therapeutic agents. See, e.g., Crooke and Lebleu (eds) (1993) *Antisense Research Applications* CRC Press; and, Sanghvi and Cook (eds) (1994) *Carbohydrate modifications in Antisense Research* ACS Symp. Ser. #580 ACS, Washington DC. Very stable hybrids are formed between nucleic acids and probes comprised of peptide nucleic acids, in which the entire sugar-phosphate backbone has been replaced with a polyamide structure.

Another important factor which sometimes affects the use of oligonucleotide probe arrays is the nature of the target nucleic acid. Oligodeoxynucleotide probes can hybridize to DNA and RNA targets with different affinity and specificity. For example, probe sequences containing long "runs" of consecutive deoxyadenosine residues form less stable hybrids with complementary RNA sequences than with the complementary DNA sequences. Substitution of dA in the probe with either 2,6-diaminopurine deoxyriboside, or 2'-alkoxy- or 2'-fluoro-dA enhances hybridization with RNA targets.

Internal structure within nucleic acid probes or the targets also influences hybridization efficiency. For example, GC-rich sequences, and sequences containing "runs" of consecutive G residues frequently self-associate to form higher-order structures, and this can inhibit their binding to complementary sequences. See, Zimmermann et al. (1975) *J. Mol Biol* 92: 181; Kim (1991) *Nature* 351: 331; Sen and Gilbert (1988) *Nature* 335: 364; and Sunquist and Klug (1989) *Nature* 342: 825. These structures are selectively destabilized by the substitution of one or more guanine residues with one or more of the following purines or purine analogs: 7-deazaguanine, 8-aza-7-deazaguanine, 2-aminopurine, 1H-purine, and hypoxanthine, in order to enhance hybridization.

Modified nucleic acids and nucleic acid analogs can also be used to improve the chemical stability of probe arrays. For example, certain processes and conditions that are useful for either the fabrication or subsequent use of the arrays, may not be compatible with standard oligonucleotide chemistry, and alternate chemistry can be employed to overcome these problems. For example, exposure to acidic conditions will cause depurination of purine nucleotides, ultimately resulting in chain cleavage and overall degradation of the probe array. In this case, adenine and guanine are replaced with 7-deazaadenine and 7-deazaguanine, respectively, in order to stabilize the oligonucleotide probes towards acidic conditions which are used during the manufacture or use of the arrays.

Base, phosphate and sugar modifications are used in combination to make highly modified oligonucleotide analogues which take advantage of the properties of each of the various modifications. For example, oligonucleotides which have higher binding affinities for complementary sequences than their unmodified counterparts (e.g., 2'-O-methyl-, 2'-O-propyl-, and 2'-O-allyl oligonucleotides) can be incorporated into oligonucleotides with modified bases (deazaguanine, 8-aza-7-deazaguanine, 2-aminopurine, 1H-purine, hypoxanthine and the like) with non-ionic methylphosphonate linkages or neutral or cationic phosphoramidate linkages, resulting in additive stabilization of duplex formation between the oligonucleotide and a target nucleic acid. For instance, one preferred oligonucleotide comprises a 2'-O-methyl-2,6-diaminopurineriboside phosphorothioate. Similarly, any of the modified bases described herein can be incorporated into peptide nucleic acids, in which the entire

sugar-phosphate backbone has been replaced with a polyamide structure.

Thermal equilibrium studies, kinetic "on-rate" studies, and sequence specificity analysis is optionally performed for any target oligonucleotide and probe or probe analogue. The data obtained shows the behavior of the analogues upon duplex formation with target oligonucleotides. Altered duplex stability conferred by using oligonucleotide analogue probes are ascertained by following, e.g., fluorescence signal intensity of oligonucleotide analogue arrays hybridized with a target oligonucleotide over time. The data allow optimization of specific hybridization conditions at, e.g., room temperature (for simplified diagnostic applications).

Another way of verifying altered duplex stability is by following the signal intensity generated upon hybridization with time. Previous experiments using DNA targets and DNA chips have shown that signal intensity increases with time, and that the more stable duplexes generate higher signal intensities faster than less stable duplexes. The signals reach a plateau or "saturate" after a certain amount of time due to all of the binding sites becoming occupied. These data allow for optimization of hybridization, and determination of equilibration conditions at a specified temperature.

Graphs of signal intensity and base mismatch positions are plotted and the ratios of perfect match versus mismatches calculated. This calculation shows the sequence specific properties of nucleotide analogues as probes. Perfect match/mismatch ratios greater than 4 are often desirable in an oligonucleotide diagnostic assay because, for a diploid genome, ratios of 2 have to be distinguished (e.g., in the case of a heterozygous trait or sequence).

Target Nucleic Acids Which Comprise Nucleotide Analogues

Modified nucleotides and nucleotide analogues are incorporated synthetically or enzymatically into DNA or RNA target nucleic acids for hybridization analysis to oligonucleotide arrays. The incorporation of nucleotide analogues in the target optimizes the hybridization of the target in terms of sequence specificity and/or the overall affinity of binding to oligonucleotide and oligonucleotide analogue probe arrays. The use of nucleotide analogues in either the oligonucleotide array or the target nucleic acid, or both, improves optimizability of hybridization interactions. Examples of useful nucleotide analogues which are substituted for naturally occurring nucleotides include 7-deazaguanosine, 2,6-diaminopurine nucleotides, 5-propynyl and other 5-substituted pyrimidine nucleotides, 2'-fluoro and 2'-methoxy -2'-deoxynucleotides and the like.

These nucleotide analogues are incorporated into nucleic acids using the synthetic methods described supra, or using DNA or RNA polymerases. The nucleotide analogues are preferably incorporated into target nucleic acids using in vitro amplification methods such as PCR, LCR, Q β -replicase expansion, in vitro transcription (e.g., nick translation or random-primer transcription) and the like. Alternatively, the nucleotide analogues are optionally incorporated into cloned nucleic acids by culturing a cell which comprises the cloned nucleic acid in media which includes a nucleotide analogue.

Similar to the use of nucleotide analogues in probe arrays, 7-deazaguanosine is used in target nucleic acids to substitute for G/dG to enhance target hybridization by reducing secondary structure in sequences containing runs of poly-G/dG. 6-diaminopurine nucleotides substitute for A/dA to enhance target hybridization through enhanced H-bonding to T or U rich probes. 5-propynyl and other 5-substituted pyrimidine

nucleotides substitute for natural pyrimidines to enhance target hybridization to certain purine rich probes. 2'-fluoro and 2'-methoxy-2'-deoxynucleotides substitute for natural nucleotides to enhance target hybridization to similarly substituted probe sequences.

Synthesis of 5'-photoprotected 2'-O alkyl ribonucleotide analogues

The light-directed synthesis of complex arrays of nucleotide analogues on a glass surface is achieved by derivatizing cyanoethyl phosphoramidite nucleotides and nucleotide analogues (e.g., nucleoside analogues of uridine, thymidine, cytidine, adenosine and guanosine, with phosphates) with, for example, the photolabile MeNPoc group in the 5'-hydroxyl position instead of the usual dimethoxytrityl group. See, application SN PCT/US94/12305.

Specific base-protected 2'-O alkyl nucleosides are commercially available, from, e.g., Chem Genes Corp. (MA). The photolabile MeNPoc group is added to the 5'-hydroxyl position followed by phosphitylation to yield cyanoethyl phosphoramidite monomers. Commercially available nucleosides are optionally modified (e.g., by 2'-O-alkylation) to create nucleoside analogues which are used to generate oligonucleotide analogues.

Modifications to the above procedures are used in some embodiments to avoid significant addition of MeNPoc to the 3'-hydroxyl position. For instance, in one embodiment, a 2'-O-methyl ribonucleotide analogue is reacted with DMT-Cl {di(p-methoxyphenyl)phenylchloride} in the presence of pyridine to generate a 2'-O-methyl-5'-O-DMT ribonucleotide analogue. This allows for the addition of TBDMS to the 3'-O of the ribonucleoside analogue by reaction with TBDMS-Triflate (t-butyldimethylsilyltrifluoromethanesulfonate) in the presence of triethylamine in THF (tetrahydrofuran) to yield a 2'-O-methyl-3'-O-TBDMS-5'-O-DMT ribonucleotide base analogue. This analogue is treated with TCAA (trichloroacetic acid) to cleave off the DMT group, leaving a reactive hydroxyl group at the 5' position. MeNPoc is then added to the oxygen of the 5' hydroxyl group using MeNPoc-Cl in the presence of pyridine. The TBDMS group is then cleaved with F⁻ (e.g., NaF) to yield a ribonucleotide base analogue with a MeNPoc group attached to the 5' oxygen on the nucleotide analogue. If appropriate, this analogue is phosphitylated to yield a phosphoramidite for oligonucleotide analogue synthesis. Other nucleosides or nucleoside analogues are protected by similar procedures.

Synthesis of Oligonucleotide Analogue Arrays on Chips

Other than the use of photoremovable protecting groups, the nucleoside coupling chemistry used in VLSIPS™ technology for synthesizing oligonucleotides and oligonucleotide analogues on chips is similar to that used for oligonucleotide synthesis. The oligonucleotide is typically linked to the substrate via the 3'-hydroxyl group of the oligonucleotide and a functional group on the substrate which results in the formation of an ether, ester, carbamate or phosphate ester linkage. Nucleotide or oligonucleotide analogues are attached to the solid support via carbon-carbon bonds using, for example, supports having (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide as the solid support). Siloxane bonds with the surface of the support are formed in one embodiment via reactions of surface attaching portions bearing trichlorosilyl or trialkoxysilyl groups. The surface attaching groups have a site for attachment of the oligonucleotide analogue portion. For example, groups which are suitable for attachment include amines, hydroxyl, thiol, and

carboxyl. Preferred surface attaching or derivitizing portions include aminoalkylsilanes and hydroxyalkylsilanes. In particularly preferred embodiments, the surface attaching portion of the oligonucleotide analogue is either bis(2-hydroxyethyl)-aminopropyltriethoxysilane, n-(3-triethoxysilylpropyl)-4-hydroxybutylamide, aminopropyltriethoxysilane or hydroxypropyltriethoxysilane.

The oligoribonucleotides generated by synthesis using ordinary ribonucleotides are usually base labile due to the presence of the 2'-hydroxyl group. 2'-O-methyloligoribonucleotides (2'-OMeORNs), analogues of RNA where the 2'-hydroxyl group is methylated, are DNAse and RNAse resistant, making them less base labile. Sproat, B. S., and Lamond, A. I. in *Oligonucleotides and Analogues: A Practical Approach*, edited by F. Eckstein, New York: IRL Press at Oxford University Press, 1991, pp. 49-86, incorporated herein by reference for all purposes, have reported the synthesis of mixed sequences of 2'-O-Methoxyoligoribonucleotides (2'-O-MeORNs) using dimethoxytrityl phosphoramidite chemistry. These 2'-O-MeORNs display greater binding affinity for complementary nucleic acids than their unmodified counterparts.

Other embodiments of the invention provide mechanical means to generate oligonucleotide analogues. These techniques are discussed in co-pending application Ser. No. 07/796,243, filed Nov. 22, 1991, which is incorporated herein by reference in its entirety for all purposes. Essentially, oligonucleotide analogue reagents are directed over the surface of a substrate such that a predefined array of oligonucleotide analogues is created. For instance, a series of channels, grooves, or spots are formed on or adjacent to a substrate. Reagents are selectively flowed through or deposited in the channels, grooves, or spots, forming an array having different oligonucleotides and/or oligonucleotide analogues at selected locations on the substrate.

Detection of Hybridization

In one embodiment, hybridization is detected by labeling a target with, e.g., fluorescein or other known visualization agents and incubating the target with an array of oligonucleotide analogue probes. Upon duplex formation by the target with a probe in the array (or triplex formation in embodiments where the array comprises unimolecular double-stranded probes), the fluorescein label is excited by, e.g., an argon laser and detected by viewing the array, e.g., through a scanning confocal microscope.

Sequencing by hybridization

Current sequencing methodologies are highly reliant on complex procedures and require substantial manual effort. Conventional DNA sequencing technology is a laborious procedure requiring electrophoretic size separation of labeled DNA fragments. An alternative approach involves a hybridization strategy carried out by attaching target DNA to a surface. The target is interrogated with a set of oligonucleotide probes, one at a time (see, application SN PCT/US94/12305).

A preferred method of oligonucleotide probe array synthesis involves the use of light to direct the synthesis of oligonucleotide analogue probes in high-density, miniaturized arrays. Matrices of spatially-defined oligonucleotide analogue probe arrays were generated. The ability to use these arrays to identify complementary sequences was demonstrated by hybridizing fluorescent labeled oligonucleotides to the matrices produced.

Oligonucleotide analogue arrays are used, e.g., to study sequence specific hybridization of nucleic acids, or protein-

nucleic acid interactions. Oligonucleotide analogue arrays are used to define the thermodynamic and kinetic rules governing the formation and stability of oligonucleotide and oligonucleotide analogue complexes.

Oligonucleotide analogue Probe Arrays and Libraries

The use of oligonucleotide analogues in probe arrays provides several benefits as compared to standard oligonucleotide arrays. For instance, as discussed supra, certain oligonucleotide analogues have enhanced hybridization characteristics to complementary nucleic acids as compared with oligonucleotides made of naturally occurring nucleotides. One primary benefit of enhanced hybridization characteristics is that oligonucleotide analogue probes are optionally shorter than corresponding probes which do not include nucleotide analogues.

Standard oligonucleotide probe arrays typically require fairly long probes (about 15–25 nucleotides) to achieve strong binding to target nucleic acids. The use of such long probes is disadvantageous for two reasons. First, the longer the probe, the more synthetic steps must be performed to make the probe and any probe array comprising the probe. This increases the cost of making the probes and arrays. Furthermore, as each synthetic step results in less than 100% coupling for every nucleotide, the quality of the probes degrades as they become longer. Secondly, short probes provide better mis-match discrimination for hybridization to a target nucleic acid. This is because a single base mismatch for a short probe-target hybridization is less destabilizing than a single mismatch for a long probe-target hybridization. Thus, it is harder to distinguish a single probe-target mismatch when the probe is a 20-mer than when the probe is an 8-mer. Accordingly, the use of short oligonucleotide analogue probes reduces costs and increases mismatch discrimination in probe arrays.

The enhanced hybridization characteristics of oligonucleotide analogues also allows for the creation of oligonucleotide analogue probe arrays where the probes in the arrays have substantial secondary structure. For instance, the oligonucleotide analogue probes are optionally configured to be fully or partially double stranded on the array. The probes are optionally complexed with complementary nucleic acids, or are optionally unimolecular oligonucleotides with self-complementary regions. Libraries of diverse double-stranded oligonucleotide analogue probes are used, for example, in screening studies to determine binding affinity of nucleic acid binding proteins, drugs, or oligonucleotides (e.g., to examine triple helix formation). Specific oligonucleotide analogues are known to be conducive to the formation of unusual secondary structure. See, Durland (1995) *Bioconjugate Chem.* 6: 278–282. General strategies for using unimolecular double-stranded oligonucleotides as probes and for library generation is described in application Ser. No. 08/327,687, and similar strategies are applicable to oligonucleotide analogue probes.

In general, a solid support, which optionally has an attached spacer molecule is attached to the distal end of the oligonucleotide analogue probe. The probe is attached as a single unit, or synthesized on the support or spacer in a monomer by monomer approach using the VLSIPS™ or mechanical partitioning methods described supra. Where the oligonucleotide analogue arrays are fully double-stranded, oligonucleotides (or oligonucleotide analogues) complementary to the probes on the array are hybridized to the array.

In some embodiments, molecules other than oligonucleotides, such as proteins, dyes, co-factors, linkers

and the like are incorporated into the oligonucleotide analogue probe, or attached to the distal end of the oligomer, e.g., as a spacing molecule, or as a probe or probe target. Flexible linkers are optionally used to separate complementary portions of the oligonucleotide analogue.

The present invention also contemplates the preparation of libraries of oligonucleotide analogues having bulges or loops in addition to complementary regions. Specific RNA bulges are often recognized by proteins (e.g., TAR RNA is recognized by the TAT protein of HIV). Accordingly, libraries of oligonucleotide analogue bulges or loops are useful in a number of diagnostic applications. The bulge or loop can be present in the oligonucleotide analogue or linker portions.

Unimolecular analogue probes can be configured in a variety of ways. In one embodiment, the unimolecular probes comprise linkers, for example, where the probe is arranged according to the formula $Y-L^1-X^1-L^2-X^2$, in which Y represents a solid support, X^1 and X^2 represent a pair of complementary oligonucleotides or oligonucleotide analogues, L^1 represents a bond or a spacer, and L^2 represents a linking group having sufficient length such that X^1 and X^2 form a double-stranded oligonucleotide. The general synthetic and conformational strategy used in generating the double-stranded unimolecular probes is similar to that described in co-pending application Ser. No. 08/327,687, except that any of the elements of the probe (L^1 , X^1 , L^2 and X^2) comprises a nucleotide or an oligonucleotide analogue. For instance, in one embodiment X^1 is an oligonucleotide analogue.

The oligonucleotide analogue probes are optionally arranged to present a variety of moieties. For example, structural components are optionally presented from the middle of a conformationally restricted oligonucleotide analogue probe. In these embodiments, the analogue probes generally have the structure X^1-Z-X^2 wherein X^{11} and X^{12} are complementary oligonucleotide analogues and Z is a structural element presented away from the surface of the probe array. Z can include an agonist or antagonist for a cell membrane receptor, a toxin, venom, viral epitope, hormone, peptide, enzyme, cofactor, drug, protein, antibody or the like.

General tiling strategies for detection of a Polymorphism in a target oligonucleotide

In diagnostic applications, oligonucleotide analogue arrays (e.g., arrays on chips, slides or beads) are used to determine whether there are any differences between a reference sequence and a target oligonucleotide, e.g., whether an individual has a mutation or polymorphism in a known gene. As discussed supra, the oligonucleotide target is optionally a nucleic acid such as a PCR amplicon which comprises one or more nucleotide analogues. In one embodiment, arrays are designed to contain probes exhibiting complementarity to one or more selected reference sequence whose sequence is known. The arrays are used to read a target sequence comprising either the reference sequence itself or variants of that sequence. Any polynucleotide of known sequence is selected as a reference sequence. Reference sequences of interest include sequences known to include mutations or polymorphisms associated with phenotypic changes having clinical significance in human patients. For example, the CFTR gene and P53 gene in humans have been identified as the location of several mutations resulting in cystic fibrosis or cancer respectively. Other reference sequences of interest include those that serve to identify pathogenic microorganisms and/or are the site of mutations by which such microorganisms acquire

drug resistance (e.g., the HIV reverse transcriptase gene for HIV resistance). Other reference sequences of interest include regions where polymorphic variations are known to occur (e.g., the D-loop region of mitochondrial DNA). These reference sequences also have utility for, e.g., forensic, cladistic, or epidemiological studies.

Other reference sequences of interest include those from the genome of pathogenic viruses (e.g., hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, CMV, and Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus. Other reference sequences of interest are from genomes or episomes of pathogenic bacteria, particularly regions that confer drug resistance or allow phylogenetic characterization of the host (e.g., 16S rRNA or corresponding DNA). For example, such bacteria include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumococci, meningococci and gonococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria. Other reference sequences of interest include those in which mutations result in the following autosomal recessive disorders: sickle cell anemia, β -thalassemia, phenylketonuria, galactosemia, Wilson's disease, hemochromatosis, severe combined immunodeficiency, alpha-1-antitrypsin deficiency, albinism, alkaptonuria, lysosomal storage diseases and Ehlers-Danlos syndrome. Other reference sequences of interest include those in which mutations result in X-linked recessive disorders: hemophilia, glucose-6-phosphate dehydrogenase, agammaglobulinemia, diabetes insipidus, Lesch-Nyhan syndrome, muscular dystrophy, Wiskott-Aldrich syndrome, Fabry's disease and fragile X-syndrome. Other reference sequences of interest includes those in which mutations result in the following autosomal dominant disorders: familial hypercholesterolemia, polycystic kidney disease, Huntington's disease, hereditary spherocytosis, Marfan's syndrome, von Willebrand's disease, neurofibromatosis, tuberous sclerosis, hereditary hemorrhagic telangiectasia, familial colonic polyposis, Ehlers-Danlos syndrome, myotonic dystrophy, muscular dystrophy, osteogenesis imperfecta, acute intermittent porphyria, and von Hippel-Lindau disease.

Although an array of oligonucleotide analogue probes is usually laid down in rows and columns for simplified data processing, such a physical arrangement of probes on the solid substrate is not essential. Provided that the spatial location of each probe in an array is known, the data from the probes is collected and processed to yield the sequence of a target irrespective of the physical arrangement of the probes on, e.g., a chip. In processing the data, the hybridization signals from the respective probes is assembled into any conceptual array desired for subsequent data reduction, whatever the physical arrangement of probes on the substrate.

In one embodiment, a basic tiling strategy provides an array of immobilized probes for analysis of a target oligonucleotide showing a high degree of sequence similarity to one or more selected reference oligonucleotide (e.g., detection of a point mutation in a target sequence). For instance, a first probe set comprises a plurality of probes exhibiting perfect complementarity with a selected reference oligonucleotide. The perfect complementarity usually exists

throughout the length of the probe. However, probes having a segment or segments of perfect complementarity that is/are flanked by leading or trailing sequences lacking complementarity to the reference sequence can also be used. Within a segment of complementarity, each probe in the first probe set has at least one interrogation position that corresponds to a nucleotide in the reference sequence. The interrogation position is aligned with the corresponding nucleotide in the reference sequence when the probe and reference sequence are aligned to maximize complementarity between the two. If a probe has more than one interrogation position, each corresponds with a respective nucleotide in the reference sequence. The identity of an interrogation position and corresponding nucleotide in a particular probe in the first probe set cannot be determined simply by inspection of the probe in the first set. An interrogation position and corresponding nucleotide is defined by the comparative structures of probes in the first probe set and corresponding probes from additional probe sets.

For each probe in the first set, there are, for purposes of the present illustration, multiple corresponding probes from additional probe sets. For instance, there are optionally probes corresponding to each nucleotide of interest in the reference sequence. Each of the corresponding probes has an interrogation position aligned with that nucleotide of interest. Usually, the probes from the additional probe sets are identical to the corresponding probe from the first probe set with one exception. The exception is that at the interrogation position, which occurs in the same position in each of the corresponding probes from the additional probe sets. This position is occupied by a different nucleotide in the corresponding probe sets. Other tiling strategies are also employed, depending on the information to be obtained.

The probes are oligonucleotide analogues which are capable of hybridizing with a target nucleic sequence by complementary base-pairing. Complementary base pairing includes sequence-specific base pairing, which comprises, e.g., Watson-Crick base pairing or other forms of base pairing such as Hoogsteen base pairing. The probes are attached by any appropriate linkage to a support. 3' attachment is more usual as this orientation is compatible with the preferred chemistry used in solid phase synthesis of oligonucleotides and oligonucleotide analogues (with the exception of, e.g., analogues which do not have a phosphate backbone, such as peptide nucleic acids).

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. A variety of parameters can be changed or modified to yield essentially similar results.

One approach to enhancing oligonucleotide hybridization is to increase the thermal stability (T_m) of the duplex formed between the target and the probe using oligonucleotide analogues that are known to increase T_m 's upon hybridization to DNA. Enhanced hybridization using oligonucleotide analogues is described in the examples below, including enhanced hybridization in oligonucleotide arrays.

Example 1

Solution oligonucleotide melting T_m

The T_m of 2'-O-methyl oligonucleotide analogues was compared to the T_m for the corresponding DNA and RNA sequences in solution. In addition, the T_m of 2'-O-methyl oligonucleotide:DNA, 2'-O-methyl oligonucleotide:RNA and RNA:DNA duplexes in solution was also determined.

The T_m was determined by varying the sample temperature and monitoring the absorbance of the sample solution at 260 nm. The oligonucleotide samples were dissolved in a 0.1M NaCl solution with an oligonucleotide concentration of 2 μ M. Table 1 summarizes the results of the experiment. The results show that the hybridization of DNA in solution has approximately the same T_m as the hybridization of DNA with a 2'-O-methyl-substituted oligonucleotide analogue. The results also show that the T_m for the 2'-O-methyl-substituted oligonucleotide duplex is higher than that for the corresponding RNA:2'-O-methyl-substituted oligonucleotide duplex, which is higher than the T_m for the corresponding DNA:DNA or RNA:DNA duplex.

TABLE 1

Solution Oligonucleotide Melting Experiments		
(+) = Target Sequence (5'-CTGAACGGTAGCATCTTGAC-3')(SEQ ID NO: 6)* (-) = Complementary Sequence (5'-GTCGAAGATGCTACCGTTCAAG-3')(SEQ ID NO: 7)*		
Type of Oligonucleotide, Target Sequence (+)	Type of Oligonucleotide, Complementary Sequence (+)	T_m ($^{\circ}$ C.)
DNA(+)	DNA(-)	61.6
DNA(+)	2'OMe(-)	58.6
2'OMe(+)	DNA(-)	61.6
2'OMe(+)	2'OMe(-)	78.0
RNA(+)	DNA(-)	58.2
RNA(+)	2'OMe(-)	73.6

*T refers to thymine for the DNA oligonucleotides, or uracil for the RNA oligonucleotides.

Example 2

Array hybridization experiments with DNA chips and oligonucleotide analogue targets

A variable length DNA probe array on a chip was designed to discriminate single base mismatches in the 3 corresponding sequences 5'-CTGAACGGTAGCATCTTGAC-3' (SEQ ID NO:6) (DNA target), 5'-CUGAACGGUAGCAUCUUGAC-3' (SEQ ID NO:8) (RNA target) and 5'-CUGAACGGUAGCAUCUUGAC-3' (SEQ ID NO:9) (2'-O-methyl oligonucleotide target), and generated by the VLSIPSTM procedure. The Chip was designed with adjacent 12-mers and 8-mers which overlapped with the 3 target sequences as shown in Table 2.

TABLE 2

Array hybridization Experiments	
Target 1 (DNA)	5'-CTGAACGGTAGCATCTTGAC-3' (SEQ ID NO: 6)
8-mer probe (complement)	_____
12-mer probe (complement)	_____
Target 2 (RNA)	5'-CUGAACGGUAGCAUCUUGAC-3' (SEQ ID NO: 8)
8-mer probe (complement)	_____
12-mer probe (complement)	_____
Target 3 (2'-O-Me oligo)	5'-CUGAACGGUAGCAUCUUGAC-3' (SEQ ID NO: 9)
8-mer probe (complement)	_____
12-mer probe (complement)	_____

Target oligos were synthesized using standard techniques. The DNA and 2'-O-methyl oligonucleotide analogue target oligonucleotides were hybridized to the chip at a concentration of 10 nM in 5 \times SSPE at 20 $^{\circ}$ C. in sequential experiments. Intensity measurements were taken at each probe position in the 8-mer and 12-mer arrays over time. The

rate of increase in intensity was then plotted for each probe position. The rate of increase in intensity was similar for both targets in the 8-mer probe arrays, but the 12-mer probes hybridized more rapidly to the DNA target oligonucleotide.

Plots of intensity versus probe position were generated for the RNA, DNA and 2'-O-methyl oligonucleotides to ascertain mismatch discrimination. The 8-mer probes displayed similar mismatch discrimination against all targets. The 12-mer probes displayed the highest mismatch discrimination for the DNA targets, followed by the 2'-O-methyl target, with the RNA target showing the poorest mismatch discrimination.

Thermal equilibrium experiments were performed by hybridizing each of the targets to the chip for 90 minutes at 5 $^{\circ}$ C. temperature intervals. The chip was hybridized with the target in 5 \times SSPE at a target concentration of 10 nM. Intensity measurements were taken at the end of the 90 minute hybridization at each temperature point as described above. All of the targets displayed similar stability, with minimal hybridization to the 8-mer probes at 30 $^{\circ}$ C. In addition, all of the targets showed similar stability in hybridizing to the 12-mer probes. Thus, the 2'-O-methyl oligonucleotide target had similar hybridization characteristics to DNA and RNA targets when hybridized against DNA probes.

Example 3

2'-O-methyl-substituted oligonucleotide chips

DMT-protected DNA and 2'-O-methyl phosphoramidites were used to synthesize 8-mer probe arrays on a glass slide using the VLSIPSTM method. The resulting chip was hybridized to DNA and RNA targets in separate experiments. The target sequence, the sequences of the probes on the chip and the general physical layout of the chip is described in Table 3.

The chip was hybridized to the RNA and DNA targets in successive experiments. The hybridization conditions used were 10 nM target, in 5 \times SSPE. The chip and solution were heated from 20 $^{\circ}$ C. to 50 $^{\circ}$ C., with a fluorescence measurement taken at 5 degree intervals as described in SN PCT/US94/12305. The chip and solution were maintained at each temperature for 90 minutes prior to fluorescence measurements. The results of the experiment showed that DNA probes were equal or superior to 2'-O-methyl oligonucleotide analogue probes for hybridization to a DNA target, but that the 2'-O-methyl analogue oligonucleotide probes

showed dramatically better hybridization to the RNA target than the DNA probes. In addition, the 2'-O-methyl analogue oligonucleotide probes showed superior mismatch discrimination of the RNA target compared to the DNA probes. The difference in fluorescence intensity between the matched and mismatched analogue probes was greater than the difference

between the matched and mismatched DNA probes, dramatically increasing the signal-to-noise ratio. FIG. 1 displays the results graphically (FIGS. 1A and 1B). (M) and (P) indicate mismatched and perfectly matched probes, respectively. (FIGS. 1C and 1D) illustrates the fluorescence intensity versus location on an example chip for the various probes at 20° C. using DNA and RNA targets, respectively.

TABLE 3

2'-O-methyl Oligonucleotide Analogues on a Chip.	
Target Sequence (DNA):	5'-CTGAACGGTAGCATCTTGAC-3' (SEQ ID NO: 6)
Target Sequence (RNA):	5'-CUGAACGGUAGCAUCUUGAC-3' (SEQ ID NO: 8)
Matching DNA oligonucleotide probe {DNA (M)}	5'-CTTGCCAT (SEQ ID NO: 10)
Matching 2'-O-methyl oligonucleotide analogue probe {2'OMe (M)}	5'-CUUGCCAU (SEQ ID NO: 11)
DNA oligonucleotide probe with 1 base mismatch {DNA (P)}	5'-CTTGCTAT (SEQ ID NO: 12)
2'-O-methyl oligonucleotide analogue probe with 1 base mismatch {2'OMe (M)}	5'-CUUGCUAU (SEQ ID NO: 13)
SCHEMATIC REPRESENTATION OF 2'-O-METHYL/DNA CHIP	
Matching 2'-O-methyl oligonucleotide analogue probe	
2'-O-methyl oligonucleotide analogue probe with 1 base mismatch	
DNA oligonucleotide probe with 1 base mismatch	
Matching DNA oligonucleotide probe	

Example 4

Synthesis of oligonucleotide analogues

The reagent MeNPoc-Cl group reacts non-selectively with both the 5' and 3' hydroxyls on 2'-O-methyl nucleoside analogues. Thus, to generate high yields of 5'-O-MeNPoc-2'-O-methylribonucleoside analogues for use in oligonucleotide analogue synthesis, the following protection-deprotection scheme was utilized.

The protective group DMT was added to the 5'-O position of the 2'-O-methylribonucleoside analogue in the presence of pyridine. The resulting 5'-O-DMT protected analogue was reacted with TBDMS-Triflate in THF, resulting in the addition of the TBDMS group to the 3'-O of the analogue. The 5'-DMT group was then removed with TCAA to yield a free OH group at the 5' position of the 2'-O-methyl ribonucleoside analogue, followed by the addition of MeNPoc-Cl in the presence of pyridine, to yield 5'-O-MeNPoc-3'-O-TBDMS-2'-O-methyl ribonucleoside analogue. The TBDMS group was then removed by reaction with NaF, and the 3'-OH group was phosphorylated using standard techniques.

Two other potential strategies did not result in high specific yields of 5'-O-MeNPoc-2'-O-methylribonucleoside. In the first, a less reactive MeNPoc derivative was synthesized by reacting MeNPoc-Cl with N-hydroxy succinimide to yield MeNPoc-NHS. This less reactive photocleavable group (MeNPoc-NHS) was found to react exclusively with the 3' hydroxyl on the 2'-O-methylribonucleoside analogue. In the second strategy, an organotin protection scheme was used. Dibutyltin oxide was reacted with the 2'-O-methylribonucleoside analogue followed by reaction with MeNPoc. Both 5'-O-MeNPoc and 3'-O-MeNPoc 2'-O-methylribonucleoside analogues were obtained.

Example 5

Hybridization to mixed-sequence oligodeoxynucleotide probes substituted with 2-amino-2'-deoxyadenosine (D)

To test the effect of a 2-amino-2'-deoxyadenosine (D) substitution in a heterogeneous probe sequence, two 4x4

oligodeoxynucleotide arrays were constructed using VLSIPS™ methodology and 5'-O-MeNPoc-protected deoxynucleoside phosphoramidites. Each array was comprised of the following set of probes based on the sequence (3')-CATCGTAGAA-(5') (SEQ ID NO:1):

- 1.-(HEG)-(3')-CATN₁GTAGAA-(5') (SEQ ID NO:14)
- 2.-(HEG)-(3')-CATCN₂TAGAA-(5') (SEQ ID NO:15)
- 3.-(HEG)-(3')-CATCGN₃AGAA-(5') (SEQ ID NO:16)
- 4.-(HEG)-(3')-CATCGTN₄GAA-(5') (SEQ ID NO:17)

where HEG=hexaethyleneglycol linker, and N is either A,G,C or T, so that probes are obtained which contain single mismatches introduced at each of four central locations in the sequence. The first probe array was constructed with all natural bases. In the second array, 2-amino-2'-deoxyadenosine (D) was used in place of adenosine (A). Both arrays were hybridized with a 5'-fluorescein-labeled oligodeoxynucleotide target, (5')-Fl-d (CTGAACGGTAGCATCTTGAC)-(3') (SEQ ID NO:18), which contained a sequence (in bold) complementary to the base probe sequence. The hybridization conditions were: 10 nM target in 5x SSPE buffer at 22° C. with agitation. After 30 minutes, the chip was mounted on the flowcell of a scanning laser confocal fluorescence microscope, rinsed briefly with 5x SSPE buffer at 22° C., and then a surface fluorescence image was obtained.

The relative efficiency of hybridization of the target to the complementary and single-base mismatched probes was determined by comparing the average bound surface fluorescence intensity in those regions of the of the array containing the individual probe sequences. The results (FIG. 3) show that a 2-amino-2'-deoxyadenosine (D) substitution in a heterogeneous probe sequence is a relatively neutral one, with little effect on either the signal intensity or the specificity of DNA-DNA hybridization, under conditions where the target is in excess and the probes are saturated.

Example 6

Hybridization to a dA-homopolymer oligodeoxynucleotide probe substituted with 2-amino-2'-deoxyadenosine (D)

The following experiment was performed to compare the hybridization of 2'-deoxyadenosine containing homopolymer arrays with 2-amino-2'-deoxyadenosine homopolymer arrays. The experiment was performed on two 11-mer oligodeoxynucleotide probe containing arrays. Two 11-mer oligodeoxynucleotide probe sequences were synthesized on a chip using 5'-O-MeNPoc-protected nucleoside phosphoramidites and standard VLSIPS™ methodology.

The sequence of the first probe was: (HEG)-(3')-d (AAAAANAAAAA)-(5') (SEQ ID NO:19); where HEG=hexaethyleneglycol linker, and N is either A,G,C or T. The second probe was the same, except that dA was replaced by 2-amino-2'-deoxyadenosine (D). The chip was hybridized with a 5'-fluorescein-labeled oligodeoxynucleotide target, (5')-Fl-d(TTTTGTGTTT)-(3') (SEQ ID NO:20), which contained a sequence complementary to the probe sequences where N=C. Hybridization conditions were 10 nM target in 5x SSPE buffer at 22° C. with agitation. After 15 minutes, the chip was mounted on the flowcell of a scanning laser confocal fluorescence microscope, rinsed briefly with 5x SSPE buffer at 22° C. (low stringency), and a surface fluorescence image was obtained. Hybridization to the chip was continued for another 5 hours, and a surface fluorescence image was acquired again. Finally, the chip was washed briefly with 0.5x SSPE (high-stringency), then with 5x SSPE, and re-scanned.

The relative efficiency of hybridization of the target to the complementary and single-base mismatched probes was

determined by comparing the average bound surface fluorescence intensity in those regions of the of the array containing the individual probe sequences. The results (FIG. 4) indicate that substituting 2'-deoxyadenosine with 2-amino-2'-deoxyadenosine in a d(A)_n homopolymer probe sequence results in a significant enhancement in specific hybridization to a complementary oligodeoxynucleotide sequence.

Example 7

Hybridization to alternating A-T oligodeoxynucleotide probes substituted with 5-propynyl-2'-deoxyuridine (P) and 2-amino-2'-deoxyadenosine (D)

Commercially available 5'-DMT-protected 2'-deoxynucleoside/nucleoside-analog phosphoramidites (Glen Research) were used to synthesize two decanucleotide probe sequences on separate areas on a chip using a modified VLSIPS™ procedure. In this procedure, a glass substrate is initially modified with a terminal-MeNPOC-protected hexaethyleneglycol linker. The substrate was exposed to light through a mask to remove the protecting group from the linker in a checkerboard pattern. The first probe sequence was then synthesized in the exposed region using DMT-phosphoramidites with acid-deprotection cycles, and the sequence was finally capped with (MeO)₂PNIPr₂/tetrazole followed by oxidation. A second checkerboard exposure in a different (previously unexposed) region of the chip was then performed, and the second probe sequence was synthesized by the same procedure. The sequence of the first "control" probe was: -(HEG)-(3')-CGCGCCGCGC-(5') (SEQ ID NO:21); and the sequence of the second probe was one of the following:

- 1.-(HEG)-(3')-d(ATATAATATA)-(5') (SEQ ID NO:22)
- 2.-(HEG)-(3')-d(APAAPAPAPA)-(5') (SEQ ID NO:23)
- 3.-(HEG)-(3')-d(DDTDDTDDT)-(5') (SEQ ID NO:24)
- 4.-(HEG)-(3')-d(DPDPDDPDPD)-(5') (SEQ ID NO:25)

where HEG=hexaethyleneglycol linker, A=2'-deoxyadenosine, T=thymidine, D=2-amino-2'-deoxyadenosine, and P=5-propynyl-2'-deoxyuridine. Each chip was then hybridized in a solution of a fluorescein-labeled oligodeoxynucleotide target, (5')-Fluorescein-d(TATATTATAT)-(HEG)-d(GCGCGGCGCG)-(3') (SEQ ID NO:26 and SEQ ID NO:27), which is complementary to both the A/T and G/C probes. The hybridization conditions were: 10 nM target in 5× SSPE buffer at 22° C. with gentle shaking. After 3 hours, the chip was mounted on the flowcell of a scanning laser confocal fluorescence microscope, rinsed briefly with 5× SSPE buffer at 22° C., and then a surface fluorescence image was obtained. Hybridization to the chip was continued overnight (total hybridization time=20hr), and a surface fluorescence image was acquired again.

The relative efficiency of hybridization of the target to the A/T and substituted A/T probes was determined by comparing the average surface fluorescence intensity bound to those parts of the chip containing the A/T or substituted probe to the fluorescence intensity bound to the G/C control probe sequence. The results (FIG. 5) show that 5-propynyl-dU and 2-amino-dA substitution in an A/T-rich probe significantly enhances the affinity of an oligonucleotide analogue for complementary target sequences. The unsubstituted A/T-probe bound only 20% as much target as the all-G/C-probe of the same length, while the D- & P-substituted A/T probe bound nearly as much (90%) as the G/C-probe. Moreover, the kinetics of hybridization are such that, at early times, the amount of target bound to the substituted A/T probes exceeds that which is bound to the all-G/C probe.

Example 8

Hybridization to oligodeoxynucleotide probes substituted with 7-deaza-2'-deoxyguanosine (ddG) and 2'-deoxyinosine (dI)

A 16×64 oligonucleotide array was constructed using VLSIPS™ methodology, with 5'-O-MeNPOC-protected nucleoside phosphoramidites, including the analogs ddG, and dI. The array was comprised of the set of probes represented by the following sequence: -(linker)-(3')-d(A T G T T G₁ G₂ G₃ G₄ G₅ C G G G T)-(5'); (SEQ ID NO:28) where underlined bases are fixed, and the five internal deoxyguanosines (G₁₋₅) are substituted with G, ddG, dI, and T in all possible (1024 total) combinations. A complementary oligonucleotide target, labeled with fluorescein at the 5'-end: (5')-Fl-d(C A A T A C A A C C C C C G C C C A T C C)-(3') (SEQ ID NO:29), was hybridized to the array. The hybridization conditions were: 5 nM target in 6× SSPE buffer at 22° C. with shaking. After 30 minutes, the chip was mounted on the flowcell of an Affymetrix scanning laser confocal fluorescence microscope, rinsed once with 0.25 × SSPE buffer at 22° C., and then a surface fluorescence image was acquired.

The "efficiency" of target hybridization to each probe in the array is proportional to the bound surface fluorescence intensity in the region of the chip where the probe was synthesized. The relative values for a subset of probes (those containing dG→ddG and dG→dI substitutions only) are shown in FIG. 6. Substitution of guanosine with 7-deazaguanosine within the internal run of five G's results in a significant enhancement in the fluorescence signal intensity which measures hybridization. Deoxyinosine substitutions also enhance hybridization to the probe, but to a lesser extent. In this example, the best overall enhancement is realized when the dG "run" is ~40-60% substituted with 7-deaza-dG, with the substitutions distributed evenly throughout the run (i.e., alternating dG/deaza-dG).

Example 9

Synthesis of 5'-MeNPOC-2'-deoxyinosine-3'-(N,N-diisopropyl-2-cyanoethyl)phosphoramidite

2'-deoxyinosine (5.0 g, 20 mmole) was dissolved in 50 ml of dry DMF, and 100 ml dry pyridine was added and evaporated three times to dry the solution. Another 50ml pyridine was added, the solution was cooled to -20° C. under argon, and 13.8 g (50 mmole) of MeNPOC-chloride in 20 ml dry DCM was then added dropwise with stirring over 60 minutes. After 60 minutes, the cold bath was removed, and the solution was allowed to stir overnight at room temperature. Pyridine and DCM were removed by evaporation, 500 ml of ethyl acetate was added, and the solution was washed twice with water and then with brine (200 ml each). The aqueous washes were combined and back-extracted twice with ethyl acetate, and then all of the organic layers were combined, dried with Na₂SO₄, and evaporated under vacuum. The product was recrystallized from DCM to obtain 5.0 g (50% yield) of pure 5'-O-MeNPOC-2'-deoxyinosine as a yellow solid (99% purity, according to ¹H-NMR and HPLC analysis).

The MeNPOC-nucleoside (2.5 g, 5.1 mmole) was suspended in 60 ml of dry CH₃CN and phosphitylated with 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (1.65 g/1.66 ml; 5.5 mmole) and 0.47 g (2.7 mmole) of diisopropylammonium tetrazolide, according to the published procedure of Barone, et al. (*Nucleic Acids Res.* (1984) 12, 4051-61). The crude phosphoramidite was purified by flash chromatography on silica gel (90:8:2 DCM-MeOH-Et₃N), co-evaporated twice with anhydrous acetonitrile and dried under vacuum for ~24 hours to obtain 2.8 g (80%) of the pure product as a yellow solid (98% purity as determined by ¹H/³¹P-NMR and HPLC).

-continued

(ii) MOLECULE TYPE: RNA

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /note= "Target RNA sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CUGAACGGUA GCAUCUUGAC

20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "2'-O-methyl oligonucleotide"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /mod_base= cm

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 2
- (D) OTHER INFORMATION: /mod_base= um

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod_base= gm

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "2'-O-methyladenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "2'-O-methyladenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /mod_base= cm

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /mod_base= gm

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /mod_base= gm

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /mod_base= um

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 10
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "2'-O-methyladenosine"

-continued

- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 11
 (D) OTHER INFORMATION: /mod_base= gm
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 12
 (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 13
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "2'-O-methyladenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 14
 (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 15
 (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 16
 (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 17
 (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 18
 (D) OTHER INFORMATION: /mod_base= gm
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 19
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "2'-O-methyladenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 20
 (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION: 1..20
 (D) OTHER INFORMATION: /note= "Target 2'-O-methyl
 oligonucleotide sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

NNNNNNNNNN NNNNNNNNNN

20

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION: 1..8
 (D) OTHER INFORMATION: /note= "Matching DNA oligonucleotide
 probe"

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTTGCCAT

8

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "2'-O-methyl oligonucleotide"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 2
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 3
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 4
 - (D) OTHER INFORMATION: /mod_base= gm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 5
 - (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 6
 - (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 7
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "2'-O-methyladenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 8
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: -
 - (B) LOCATION: 1..8
 - (D) OTHER INFORMATION: /note= "Matching 2'-O-methyl oligonucleotide analogue probe"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

NNNNNNNN

8

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:

-continued

- (A) NAME/KEY: -
- (B) LOCATION: 1..8
- (D) OTHER INFORMATION: /note= "DNA oligonucleotide probe with 1 base mismatch"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTTGCTAT

8

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "2'-O-methyl oligonucleotide"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 2
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 3
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 4
 - (D) OTHER INFORMATION: /mod_base= gm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 5
 - (D) OTHER INFORMATION: /mod_base= cm
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 6
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 7
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "2'-O-methyladenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 8
 - (D) OTHER INFORMATION: /mod_base= um
- (ix) FEATURE:
 - (A) NAME/KEY: -
 - (B) LOCATION: 1..8
 - (D) OTHER INFORMATION: /note= "2'-O-methyl oligonucleotide analogue probe with 1 base mismatch"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

NNNNNNNN

8

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 10
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently
modified at the 3' phosphate group with
a hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGATGNTAN 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 10
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified
at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGATNCTAN 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 10
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified
at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAGANGCTAN 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 10
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified"

-continued

at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAGNTGCTAN

10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified
at the 5' phosphate group with a
fluorescein molecule"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

NTGAACGGTA GCATCTTGAC

20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = adenine covalently modified
at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAAAAANAAA N

11

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = thymine covalently modified
at the 5' phosphate group with a
fluorescein molecule"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

NTTTTGT T

11

-continued

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 10
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified
at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCGCCGCGN

10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "2'-deoxynucleoside/nucleoside
analogue decanucleotide probe"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: 10
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine covalently
modified at the 3' phosphate group with
a hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

NTTNTNTNTN

10

-continued

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "2'-deoxynucleoside/nucleoside analogue decanucleotide probe"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 2
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 3
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 4
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 5
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 6
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 7
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 8
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 9
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 10
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2'-deoxyadenosine covalently modified at the 3' phosphate group with a hexaethyleneglycol (HEG) linker"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

NNNNNNNNNN

10

-continued

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "2'-deoxynucleoside/nucleoside analogue decanucleotide probe"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 3
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 5
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 6
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 8
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 10
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine covalently modified at the 3' phosphate group with a hexaethyleneglycol (HEG) linker"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

NNTNTNTNTN

10

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "2'-deoxynucleoside/nucleoside analogue decanucleotide probe"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 1
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 - (A) NAME/KEY: modified_base
 - (B) LOCATION: 2
 - (D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = 5-propynyl-2'-deoxyuridine"

-continued

- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 3
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 4
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 5
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 6
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 7
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 8
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 2-amino-2'-deoxyadenosine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 9
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 5-propynyl-2'-deoxyuridine"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 10
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = 2-amino-2'-deoxyadenosine
 covalently modified at the 3'
 phosphate group with a
 hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

NNNNNNNNNN

10

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = thymine covalently modified
 at the 5' hydroxyl group with a
 fluorescein molecule"
- (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 10
 (D) OTHER INFORMATION: /mod_base= OTHER

-continued

/note= "N = thymine covalently modified
at the 3' phosphate group with a
hexaethyleneglycol (HEG) linker which is
covalently bound to the 5' phosphate
group of the 5' guanine (N in pos. 1) of
SEQ ID NO:27"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

NATATTATAN

10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: modified_base
(B) LOCATION: 1
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = guanine covalently modified
at the 5' phosphate group with a
hexaethyleneglycol (HEG) linker which is
covalently bound to the 3' phosphate
group of the 3' thymine (N in pos. 10)
of SEQ ID NO:26"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

NCGGCGCGCG

10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: modified_base
(B) LOCATION: 6..10
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = guanine (G),
2',3'-dideoxyguanine (ddG),
2'-deoxyinosine (dI) or thymine (T)"

(ix) FEATURE:

(A) NAME/KEY: modified_base
(B) LOCATION: 15
(D) OTHER INFORMATION: /mod_base= OTHER
/note= "N = cytosine covalently modified
at the 5' phosphate group with a
hexaethyleneglycol (HEG) linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TGGGCNNNNN TTGTN

15

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

ks(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: modified_base
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /mod_base= OTHER
 /note= "N = cytosine covalently modified
 at the 5' phosphate group with a
 fluorescein molecule"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

NAATACAACC CCCGCCCATC C

21

What is claimed is:

1. A composition for analyzing interactions between oligonucleotide targets and oligonucleotide probes comprising an array of a plurality of oligonucleotide analogue probes having different sequences, wherein said oligonucleotide analogue probes are coupled to a solid substrate at known locations and wherein said plurality of oligonucleotide analogue probes are selected to bind to complementary oligonucleotide targets with a similar hybridization stability across the array.

2. The composition of claim 1, wherein at least one of said oligonucleotide analogue probes is selected to maintain hybridization specificity or mismatch discrimination with said complementary oligonucleotide targets.

3. The composition of claim 1, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

4. The composition of claim 1, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

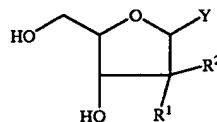
5. The composition of claim 2, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

6. The composition of claim 2, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

7. The composition of claims 1-5 or 6, wherein said solid substrate is selected from the group consisting of silica, polymeric materials, glass, beads, chips, and slides.

8. The composition of claims 1-5 or 6, wherein said composition comprises an array of oligonucleotide analogue probes 5 to 20 nucleotides in length.

9. The composition of claims 1-5 or 6, wherein said array of oligonucleotide analogue probes comprises a nucleoside analogue with the formula



wherein:

the nucleoside analogue is not a naturally occurring DNA or RNA nucleoside;

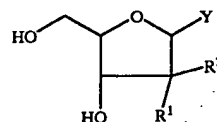
R¹ is selected from the group consisting of hydrogen, methyl, hydroxyl, alkoxy, alkylthio, halogen, cyano, and azido;

R² is selected from the group consisting of hydrogen, methyl, hydroxyl, alkoxy, alkylthio, halogen, cyano, and azido;

Y is a heterocyclic moiety;

and wherein said nucleoside analogue is incorporated into the oligonucleotide analogue by attachment to a 3' hydroxyl of the nucleoside analogue, to a 5' hydroxyl of the nucleoside analogue, or both the 3' nucleoside and the 5' hydroxyl of the nucleoside analogue.

10. The composition of claims 1-5 or 6, wherein said array of oligonucleotide analogue probes comprises a nucleoside analogue with the formula



wherein:

the nucleoside analogue is not a naturally occurring DNA or RNA nucleoside;

R¹ is selected from the group consisting of hydrogen, hydroxyl, methyl, methoxy, ethoxy, propoxy, allyloxy, propargyloxy, Fluorine, Chlorine, and Bromine;

R² is selected from the group consisting of hydrogen, hydroxyl, methyl, methoxy, ethoxy, propoxy, allyloxy, propargyloxy, Fluorine, Chlorine, and Bromine; and Y is a base selected from the group consisting of purines, purine analogues pyrimidines, pyrimidine analogues, 3-nitropyrrole and 5-nitroindole;

and wherein said nucleoside analogue is incorporated into the oligonucleotide analogue by attachment to a 3'

hydroxyl of the nucleoside analogue, to a 5' hydroxyl of the nucleoside analogue, or both the 3' nucleoside and the 5' hydroxyl of the nucleoside analogue.

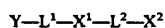
11. The composition of claims 1-5 or 6, wherein each probe of said plurality of oligonucleotide analogue probes has at least one oligonucleotide analogue, and wherein at least one of said oligonucleotide analogues comprises a peptide nucleic acid.

12. The composition of claims 1-5 or 6, wherein at least one of said plurality of oligonucleotide analogue probes said array of oligonucleotide analogue probes is resistant to RNAase A.

13. The composition of claims 1-5 or 6, wherein said solid substrate is attached to over 1000 different oligonucleotide analogue probes.

14. The composition of claims 1-5 or 6, wherein each probe of said plurality of oligonucleotide analogue probes has at least one oligonucleotide analogue, and wherein at least one of said oligonucleotide analogues comprises 2'-O-methyl nucleotides.

15. The composition of claims 1-5 or 6, wherein said array of oligonucleotide analogue probes and said solid substrate comprises a plurality of different oligonucleotide analogue probes, each oligonucleotide analogue probes having the formula:



wherein,

Y is a solid substrate;

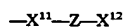
X¹ and X² are complementary oligonucleotides containing at least one nucleotide analogue;

L¹ is a spacer;

L² is a linking group having sufficient length such that X¹ and X² form a double-stranded oligonucleotide.

16. The composition of claim 15, wherein said composition comprises a library of unimolecular double-stranded oligonucleotide analogue probes.

17. The composition of claims 1-5 or 6, wherein said array of oligonucleotide analogue probes comprises a conformationally restricted array of oligonucleotide analogue probes with the formula:



wherein X¹¹ and X¹² are complementary oligonucleotides or oligonucleotide analogues and Z is a presented moiety.

18. The composition of claims 1-5 or 6, wherein each probe of said plurality of oligonucleotide analogue probes has at least one oligonucleotide analogue, and wherein at least one of said oligonucleotide analogues comprises a nucleotide with a base selected from the group of bases consisting of 5-propynyluracil, 5-propynylcytosine, 2-aminoadenine, 7-deazaguanine, 2-aminopurine, 8-aza-7-deazaguanine, 1H-purine, and hypoxanthine.

19. The composition of claims 1-5 or 6, wherein said plurality of oligonucleotide analogue probes are coupled to said solid substrate by light-directed chemical coupling.

20. The composition of claim 19, wherein said solid substrate is derivitized with a silane reagent prior to synthesis of said plurality of oligonucleotide analogue probes.

21. The composition of claims 1-5 or 6, wherein said plurality of oligonucleotide analogue probes are coupled to said solid substrate by flowing oligonucleotide analogue reagents over known locations of the solid substrate.

22. The composition of claim 21, wherein said solid substrate is derivitized with a silane reagent prior to synthesis of said plurality of oligonucleotide analogue probes.

23. The composition of claims 1-5 or 6, wherein at least one of plurality of said oligonucleotide analogue probes forms a first duplex with a target oligonucleotide sequence, wherein said oligonucleotide analogue probe has a corresponding oligonucleotide sequence that forms a second duplex with said target oligonucleotide sequence, wherein said second duplex is rich in A-T or G-C nucleotide pairs, and wherein said oligonucleotide analogue probe has at least one nucleotide analogue in place of an A, T, G, or C nucleotide of said corresponding oligonucleotide sequence at a position within said oligonucleotide analogue probe such that said first duplex has an increased hybridization stability than said second duplex.

24. The composition of claim 23, wherein said oligonucleotide analogue probe contains fewer bases than said corresponding oligonucleotide sequence.

25. The composition of claims 1-5 or 6, wherein said oligonucleotide analogue probe forms a first duplex with a target oligonucleotide sequence, wherein said oligonucleotide analogue probe has a corresponding oligonucleotide sequence that forms a second duplex with said target polynucleotide sequence, and wherein said oligonucleotide analogue probe is shorter than said corresponding polynucleotide sequence.

26. A composition for analyzing the interaction between an oligonucleotide target and an oligonucleotide probe comprising an array of a plurality of oligonucleotide probes having different sequences hybridized to complementary oligonucleotide analogue targets, wherein said oligonucleotide analogue targets bind to complementary oligonucleotide probes with a similar hybridization stability across the array.

27. The composition of claim 26, wherein at least one of said oligonucleotide analogue target is selected to maintain hybridization specificity or mismatch discrimination with said complementary oligonucleotide probes.

28. The composition of claim 26, wherein at least one of said oligonucleotide analogue targets has increased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

29. The composition of claim 26, wherein at least one of said oligonucleotide analogue targets has decreased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

30. The composition of claim 27, wherein at least one of said oligonucleotide analogue targets has increased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

31. The composition of claim 27, wherein at least one of said oligonucleotide analogue targets has decreased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

32. The composition of claims 26-30 or 31, wherein the oligonucleotide analogue target is a PCR amplicon.

33. The composition of claims 26-30 or 31, wherein at least one of said plurality of oligonucleotide probes comprise at least one oligonucleotide analogue.

34. The composition of claims 26-30 or 31, wherein at least one target oligonucleotide analogue acid is an RNA nucleic acid.

35. A method analyzing interactions between an oligonucleotide target and an oligonucleotide probe, comprising the steps of:

- (a). synthesizing an oligonucleotide analogue array comprising a plurality of oligonucleotide analogue probes having different sequences, wherein said oligonucleotide analogue probes are coupled to a solid substrate at known locations, said solid substrate having a surface;
- (b). exposing said oligonucleotide analogue probe array to a plurality of oligonucleotide targets under hybridization conditions such that said plurality of oligonucleotide analogue probes bind to complementary oligonucleotide targets with a similar hybridization stability across the array; and
- (c). determining whether an oligonucleotide analogue probe of said oligonucleotide analogue probe array binds to at least one of said target nucleic acids.

36. The method in accordance of claim 35, wherein at least one of said oligonucleotide analogue probes is selected to maintain hybridization specificity or mismatch discrimination with said complementary oligonucleotide targets.

37. The method in accordance of claim 35, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

38. The method in accordance of claim 35, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

39. The method in accordance of claim 36, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

40. The method in accordance of claim 36, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

41. The method of claims 35-39 or 40, wherein said oligonucleotide target is selected from the group comprising genomic DNA, cDNA, unspliced RNA, mRNA, and rRNA.

42. The method of claims 35-39 or 40, wherein said target nucleic acid is amplified prior to said hybridization step.

43. The method of claims 35-39 or 40, wherein said plurality of oligonucleotide analogue probes is synthesized on said solid support by light-directed synthesis.

44. The method of claims 35-39 or 40, wherein said plurality of said oligonucleotide analogue probes is synthe-

sized on said solid support by causing oligonucleotide analogue synthetic reagents to flow over known locations of said solid support.

45. The method of claims 35-39 or 40, wherein said step (a). comprises the steps of:

- i). forming a plurality of channels adjacent to the surface of said substrate;
- ii). placing selected reagents in said channels to synthesize oligonucleotide analogue probes at known locations; and
- iii). repeating steps i). and ii). thereby forming an array of oligonucleotide analogue probes having different sequences at known locations on said substrate.

46. The method of claims 35-39 or 40, wherein said solid substrate is selected from the group consisting of beads, slides, and chips.

47. The method of claims 35-39 or 40, wherein said solid substrate is comprised of materials selected from the group consisting of silica, polymers and glass.

48. The method of claims 35-39 or 40, wherein the oligonucleotide analogue probes of said array are synthesized using photoremovable protecting groups.

49. The method of claims 35-39 or 40, further comprising selectively incorporating MeNPoc onto the 3' or 5' hydroxyl of at least one nucleoside analogue and selectively incorporating said nucleoside analogue into at least one of said oligonucleotide analogue probes.

50. The method of claims 35-39 or 40, wherein at least one of said oligonucleotide analogue probes is synthesized from phosphoramidite nucleoside reagents.

51. A method of detecting an oligonucleotide target, comprising enzymatically copying an oligonucleotide target using at least one nucleotide analogue, thereby producing multiple oligonucleotide analogue targets, selecting said oligonucleotide analogue targets such that said oligonucleotide analogue targets bind to the complementary oligonucleotide probes coupled to a solid surface at known locations of an array with a similar hybridization stability across the array, hybridizing the oligonucleotide analogue targets to complementary oligonucleotide probes, and detecting whether at least one of said oligonucleotide analogue targets binds to said complementary oligonucleotide acid probe.

52. The method of claim 51, wherein at least one of said oligonucleotide analogue targets is selected to maintain hybridization specificity or mismatch discrimination with said complementary oligonucleotide probes.

53. The method of claim 51, wherein at least one of said oligonucleotide analogue targets has increased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

54. The method of claim 51, wherein at least one of said oligonucleotide analogue targets has decreased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

55. The method of claim 52, wherein at least one of said oligonucleotide analogue targets has increased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

56. The method of claim 52, wherein at least one of said oligonucleotide analogue targets has decreased the thermal stability between said oligonucleotide analogue target and said complementary oligonucleotide probe as compared to an oligonucleotide target that is the perfect complement to the complementary oligonucleotide probe with which said oligonucleotide analogue target anneals.

57. The method of claims 51-55 or 56, wherein the oligonucleotide probe array comprises at least one oligonucleotide analogue probe which is complementary to at least one of said oligonucleotide analogue targets.

58. A method of making an array of oligonucleotide probes, comprising providing a plurality of oligonucleotide analogue probes having at least one oligonucleotide analogue, said oligonucleotide analogue probes having different sequences at known locations on an array, selecting the oligonucleotide analogue probes to hybridize with complementary oligonucleotide target sequences under hybridization conditions such that said oligonucleotide analogue probes bind to complementary oligonucleotide targets with a similar hybridization stability, across the array.

59. The method of claim 58, wherein at least one of said oligonucleotide analogue probes is selected to maintain hybridization specificity or mismatch discrimination with said complementary oligonucleotide targets.

60. The method of claim 58, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

61. The method of claim 58, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

62. The method of claim 59, wherein at least one of said oligonucleotide analogue probes has increased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

63. The method of claim 59, wherein at least one of said oligonucleotide analogue probes has decreased the thermal stability between said oligonucleotide analogue probe and said complementary oligonucleotide target as compared to an oligonucleotide probe that is the perfect complement to

the complementary oligonucleotide target with which said oligonucleotide analogue probe anneals.

64. The method in accordance with claims 58-62, or 63, further comprising incorporating at least one oligonucleotide analogue into at least one of the oligonucleotide analogue probes of the array to reduce or prevent the formation of secondary structure in the oligonucleotide of the array.

65. The method in accordance with claims 58-62, or 63, further comprising incorporating at least one oligonucleotide analogue into at least one of the oligonucleotide target to reduce or prevent the formation of secondary structure in the target polynucleotide sequence.

66. The method in accordance with claims 58-62, or 63, further comprising incorporating at least one oligonucleotide analogue into at least one of the oligonucleotide analogue probes of the array to create secondary structure in the oligonucleotide of the array.

67. The method in accordance with claims 58-62, or 63, further comprising incorporating a base selected from the group consisting of 5-propynyluracil, 5-propynylcytosine, 2-aminoadenine, 7-deazaguanine, 2-aminopurine, 8-aza-7-deazaguanine, 1H-purine, and hypoxanthine into the oligonucleotide analogue probes of the array.

68. The method of claim 67 further comprising selecting said at least one oligonucleotide analogue such that the oligonucleotide analogue probe is a homopolymer.

69. The method in accordance with claims 58-62, or 63, further comprising selecting said at least one oligonucleotide analogue from the group consisting essentially of oligonucleotide analogues comprising 2'-O-methyl nucleotides and oligonucleotides comprising a base selected from the group of bases consisting of 5-propynyluracil, 5-propynylcytosine, 7-deazaguanine, 2-aminoadenine, 8-aza-7-deazaguanine, 1H-purine, and hypoxanthine.

70. The method in accordance with claims 58-62 or 63, further comprising selecting said at least one oligonucleotide analogue such that oligonucleotide analogue probes comprises at least one peptide nucleic acid.

71. The method in accordance with claims 58-62, or 63, further comprising selecting said at least one oligonucleotide analogue to increase image brightness when the oligonucleotide target and the oligonucleotide analogue probe hybridize in the presence of a fluorescent indicator, in comparison to a oligonucleotide probe without oligonucleotide analogs.

72. The method in accordance with claims 58-62, or 63, further comprising providing said plurality of oligonucleotide analogue probes in an array with at least 1000 other oligonucleotide analogue probes.

* * * * *

J

This Page Blank (uspto)



US006261776B1

(12) **United States Patent**
Pirrung et al.(10) Patent No.: **US 6,261,776 B1**
(45) Date of Patent: ***Jul. 17, 2001**(54) **NUCLEIC ACID ARRAYS**(75) Inventors: **Michael C. Pirrung**, Durham, NC (US); **J. Leighton Read**; **Stephen P. A. Fodor**, both of Palo Alto, CA (US); **Lubert Stryer**, Stanford, CA (US)(73) Assignee: **Affymetrix, Inc.**, Santa Clara, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/292,455**(22) Filed: **Apr. 15, 1999****Related U.S. Application Data**

(63) Continuation of application No. 09/129,470, filed on Aug. 4, 1998, which is a continuation of application No. 08/456,598, filed on Jun. 1, 1995, which is a division of application No. 07/954,646, filed on Sep. 30, 1992, now Pat. No. 5,445,934, which is a division of application No. 07/850,356, filed on Mar. 12, 1992, now Pat. No. 5,405,783, which is a division of application No. 07/492,462, filed on Mar. 7, 1990, now Pat. No. 5,143,854, which is a continuation-in-part of application No. 07/362,901, filed on Jun. 7, 1989, now abandoned.

(51) Int. Cl.⁷ **C12Q 1/68; G01N 33/543; A61K 38/00; C07H 21/04; C07H 21/00**(52) U.S. Cl. **435/6; 435/7.92; 435/7.94; 435/795; 435/969; 435/973; 436/518; 436/527; 436/807; 436/809; 530/334; 536/24.3; 536/24.32; 536/25.32**(58) Field of Search **435/6, 7.92, 7.94, 435/7.95, 969, 973; 436/518, 527, 807, 809; 530/334; 536/24.3, 25.3, 25.32**(56) **References Cited****U.S. PATENT DOCUMENTS**

3,849,137 11/1974 Barzynski et al. 96/67

3,862,056	1/1975	Hartman	252/511
3,939,350	2/1976	Arwin et al.	250/365
4,072,576	2/1978	Arwin et al.	195/103.5 R
4,180,739	12/1979	Abu-Shumays	250/461
4,238,757	12/1980	Schenck	357/25
4,269,933	5/1981	Pazos	430/291
4,314,821	2/1982	Rice	23/230 B
4,327,073	4/1982	Huang	424/1
4,339,528	7/1982	Goldman	430/323
4,342,905	8/1982	Fujii et al.	250/201
4,373,071	2/1983	Itakura	525/375
4,405,771	9/1983	Jagur	528/266
4,444,878	4/1984	Paulus	435/7

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

2242394	3/1974	(DE)
3440141	5/1986	(DE)
3505287	3/1988	(DE)

(List continued on next page.)

OTHER PUBLICATIONSBannwarth "Gene Technology: A Challenge for a Chemist" *Chimia*, 41:302-317 (Sep. 1987).

(List continued on next page.)

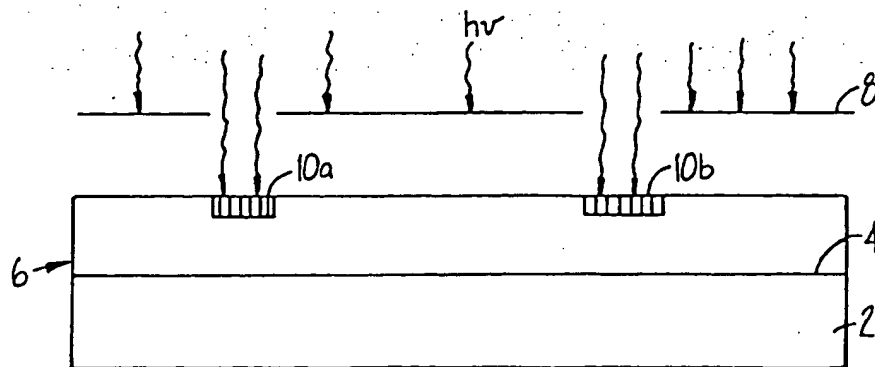
Primary Examiner—Jezia Riley

(74) Attorney, Agent, or Firm—Townsend and Townsend and Crew LLP

(57)

ABSTRACT

A method and apparatus for preparation of a substrate containing a plurality of sequences. Photoremovable groups are attached to a surface of a substrate. Selected regions of the substrate are exposed to light so as to activate the selected areas. A monomer, also containing a photoremovable group, is provided to the substrate to bind at the selected areas. The process is repeated using a variety of monomers such as amino acids until sequences of a desired length are obtained. Detection methods and apparatus are also disclosed.

39 Claims, 20 Drawing Sheets

U.S. PATENT DOCUMENTS

4,444,892	4/1984	Malmros	436/528	5,026,840	6/1991	Dattagupta et al.	536/27
4,448,534	5/1984	Wertz et al.	356/435	5,028,525	7/1991	Gray et al.	435/6
4,458,066	7/1984	Caruthers et al.	536/27	5,043,265	8/1991	Tanke et al.	435/6
4,483,920	11/1984	Gillespie et al.	435/6	5,047,524	9/1991	Andrus et al.	536/27
4,500,707	2/1985	Caruthers et al.	536/7	5,079,600	1/1992	Schnur et al.	357/4
4,516,833	5/1985	Fusek	350/162.12	5,081,584	1/1992	Omichinski et al.	364/497
4,517,338	5/1985	Urdea et al.	525/54.11	5,082,830	1/1992	Brakel et al.	514/44
4,537,861	8/1985	Elings et al.	436/518	5,091,652	2/1992	Mathies et al.	250/458.1
4,542,102	9/1985	Dattagupta et al.	435/6	5,112,962	5/1992	Letsinger et al.	536/27
4,555,490	11/1985	Merril	436/86	5,141,813	8/1992	Nelson	428/402
4,562,157	12/1985	Lowe et al.	435/291	5,143,854	9/1992	Pirung et al.	436/518
4,569,967	2/1986	Kornreich et al.	525/54.11	5,153,319	10/1992	Caruthers et al.	536/27
4,580,895	4/1986	Patel	356/39	5,192,980	3/1993	Dixon et al.	356/326
4,584,277	4/1986	Ullman	436/509	5,200,051	4/1993	Cozzette et al.	204/403
4,613,566	9/1986	Potter	435/6	5,202,231	4/1993	Drmanac et al.	435/6
4,624,915	11/1986	Schindler et al.	435/4	5,206,137	4/1993	Ip et al.	435/6
4,626,684	12/1986	Landa	250/328	5,215,882	6/1993	Bahl et al.	435/6
4,631,211	12/1986	Houghten	428/35	5,215,889	6/1993	Schultz	435/41
4,637,861	1/1987	Krull et al.	204/1	5,232,829	8/1993	Longiaru et al.	435/6
4,677,054	6/1987	White et al.	435/6	5,235,028	8/1993	Barany et al.	528/335
4,681,859	7/1987	Kramer	436/501	5,242,974	9/1993	Holmes	525/54.11
4,683,202	7/1987	Mullis	435/91	5,252,743	10/1993	Barrett et al.	548/303.7
4,689,405	8/1987	Frank et al.	536/27	5,256,549	10/1993	Urdea et al.	435/91
4,704,353	11/1987	Humphries et al.	435/4	5,258,506	11/1993	Urdea et al.	536/23.1
4,711,955	12/1987	Ward et al.	536/29	5,306,641	4/1994	Saccocio	436/85
4,713,326	12/1987	Dattagupta et al.	435/6	5,310,893	5/1994	Erlich et al.	536/24.31
4,713,347	12/1987	Mitchell et al.	436/501	5,324,633	6/1994	Fodor et al.	435/6
4,719,615	1/1988	Feyrer et al.	369/284	5,348,855	9/1994	Dattagupta et al.	435/6
4,722,906	2/1988	Guire	436/501	5,384,261	1/1995	Winkler et al.	436/518
4,728,502	3/1988	Hamill	422/116	5,405,783	4/1995	Pirung et al.	436/518
4,728,591	3/1988	Clark et al.	430/5	5,424,186	6/1995	Fodor et al.	435/6
4,731,325	3/1988	Palva et al.	435/5	5,436,327	7/1995	Southern et al.	536/25.34
4,755,458	7/1988	Rabbani et al.	435/5	5,445,934	8/1995	Fodor et al.	435/6
4,762,881	8/1988	Kauer	525/54.11	5,447,841	9/1995	Gray et al.	435/6
4,777,019	10/1988	Dandekar	422/68	5,486,452	1/1996	Gordon et al.	435/5
4,780,504	10/1988	Buendia et al.	525/54.11	5,489,507	2/1996	Chehab	435/6
4,786,170	11/1988	Groeblor	356/318	5,489,678	2/1996	Fodor et al.	536/22.1
4,786,684	11/1988	Glass	525/54.11	5,492,806	2/1996	Drmanac et al.	435/5
4,794,150	12/1988	Steel	525/54.11	5,510,270	4/1996	Fodor et al.	436/518
4,808,508	2/1989	Platzer	430/143	5,525,464	6/1996	Dramanac et al.	435/6
4,810,869	3/1989	Yabe et al.	250/501	5,527,681	6/1996	Holmes	435/6
4,811,062	3/1989	Tabata et al.	356/152	5,552,270	9/1996	Khrapko et al.	435/6
4,812,512	3/1989	Buendia et al.	525/54.11	5,556,961	9/1996	Foot et al.	536/27.1
4,820,630	4/1989	Taub	435/5	5,561,071	10/1996	Hollenberg et al.	437/1
4,822,566	4/1989	Newman	422/68	5,571,639	11/1996	Hubbell et al.	430/5
4,833,092	5/1989	Geysen	436/501	5,593,839	1/1997	Hubbell et al.	435/6
4,844,617	7/1989	Kelderman et al.	356/372	5,653,939	8/1997	Hollis et al.	422/50
4,846,552	7/1989	Veldkamp et al.	350/162.2	5,667,667	9/1997	Southern	205/687
4,849,513	7/1989	Smith et al.	536/27	5,667,972	9/1997	Drmanac et al.	435/6
4,855,225	8/1989	Fung et al.	435/6	5,695,940	12/1997	Drmanac et al.	435/6
4,865,990	9/1989	Stead et al.	435/803	5,698,393	12/1997	Macioszek et al.	435/5
4,868,103	9/1989	Stavrianopoulos et al.	435/5	5,700,637	12/1997	Southern	435/6
4,874,500	10/1989	Madou et al.	204/412	5,707,806	1/1998	Shuber	435/6
4,886,741	12/1989	Schwartz	435/5	5,744,305	4/1998	Fodor et al.	435/6
4,888,278	12/1989	Singer et al.	435/6	5,777,888	7/1998	Rine et al.	364/496
4,923,901	5/1990	Koester et al.	521/53	5,800,992	9/1998	Fodor et al.	435/6
4,925,785	5/1990	Wang et al.	435/6	5,807,522	9/1998	Brown et al.	422/50
4,946,942	8/1990	Fuller et al.	530/335	5,830,645	11/1998	Pinkel et al.	435/287.1
4,973,493	11/1990	Guire	427/2	5,843,767	12/1998	Beattie	435/6
4,979,959	12/1990	Guire	623/66	5,846,708	12/1998	Hollis et al.	435/6
4,981,783	1/1991	Augenlicht	435/6	5,871,697	2/1999	Rothberg et al.	422/68.1
4,981,985	1/1991	Kaplan et al.	556/50	5,972,619	10/1999	Drmanac et al.	435/6
4,984,100	1/1991	Takayama et al.	360/49	6,018,041	1/2000	Drmanac et al.	536/24.3
4,987,065	1/1991	Stavrianopoulos et al.	435/5	6,025,136	2/2000	Drmanac et al.	435/6
4,988,617	1/1991	Landegren et al.	435/6	6,054,270	4/2000	Southern	435/6
4,992,583	2/1991	Farnsworth	436/89				
4,994,373	2/1991	Stavrianopoulos et al.	435/6				
5,002,867	3/1991	Macevicz	435/6				
5,021,550	6/1991	Zeiger	530/334				
5,026,773	6/1991	Steel	525/54.11				

FOREIGN PATENT DOCUMENTS

046 083	2/1982 (EP)
088 636	9/1983 (EP)
103 197	3/1984 (EP)
127 0438	12/1984 (EP)

063 810 3/1986 (EP).
 194 132 9/1986 (EP).
 228 075 7/1987 (EP).
 245 662 11/1987 (EP).
 268 237 5/1988 (EP).
 281 927 9/1988 (EP).
 228 310 10/1988 (EP).
 288 310 10/1988 (EP).
 304 202 2/1989 (EP).
 307 476 3/1989 (EP).
 319 012 6/1989 (EP).
 328 256 8/1989 (EP).
 333 561 9/1989 (EP).
 337 498 10/1989 (EP).
 386 229 4/1990 (EP).
 373 203 6/1990 (EP).
 392 546 10/1990 (EP).
 173 339 1/1992 (EP).
 171 150 3/1992 (EP).
 237 362 3/1992 (EP).
 185 547 6/1992 (EP).
 260 634 6/1992 (EP).
 232 967 4/1993 (EP).
 235 726 5/1993 (EP).
 476 014 8/1994 (EP).
 225 807 10/1994 (EP).
 717 113 6/1996 (EP).
 848 067 6/1998 (EP).
 619 321 1/1999 (EP).
 2156074 3/1988 (GB).
 2559783 3/1988 (FR).
 2196476 4/1988 (GB).
 2248840 9/1992 (GB).
 49-110601 10/1974 (JP).
 60-248669 12/1985 (JP).
 63-084499 4/1988 (JP).
 63-223557 9/1988 (JP).
 1-233447 9/1989 (JP).
 WO 84/03151 8/1984 (WO).
 WO 84/03564 9/1984 (WO).
 WO 85/01051 3/1985 (WO).
 WO 86/00991 2/1986 (WO).
 WO 86/06487 11/1986 (WO).
 8810400 5/1988 (WO).
 WO 88/04777 6/1988 (WO).
 WO 89/05616 6/1989 (WO).
 WO 89/08834 9/1989 (WO).
 WO 89/11548 11/1989 (WO).
 WO 89/10977 11/1989 (WO).
 WO 89/12819 12/1989 (WO).
 WO 90/00887 2/1990 (WO).
 WO 90/15070 2/1990 (WO).
 WO 90/03382 4/1990 (WO).
 WO 90/04652 5/1990 (WO).
 WO 91/04266 4/1991 (WO).
 WO 91/07087 5/1991 (WO).
 WO 92/16655 1/1992 (WO).
 WO 92/10092 6/1992 (WO).
 WO 92/10588 6/1992 (WO).
 WO 93/02992 2/1993 (WO).
 WO 93/09668 5/1993 (WO).
 WO 88/01302 6/1993 (WO).
 WO 93/11262 6/1993 (WO).
 WO 93/22480 11/1993 (WO).
 WO 93/22546 11/1993 (WO).
 WO 95/11995 5/1995 (WO).
 WO 95/33846 12/1995 (WO).
 WO 96/23078 8/1996 (WO).
 WO 97/10365 3/1997 (WO).
 WO 97/17317 5/1997 (WO).
 WO 97/19410 5/1997 (WO).

WO 97/27317 7/1997 (WO).
 WO 97/29212 8/1997 (WO).
 WO 98/31836 7/1998 (WO).

OTHER PUBLICATIONS

Bannwarth et al. "A System for the Simultaneous Chemical Synthesis of Different DNA Fragments on Solid Support" DNA, 5:413-419 (Oct. 1986).
 Sequencing by Hybridization Workshop, listing of participants and workshop presentation summaries (1991).
 "A Sequencing Reality Check," *Science* 242:1245 (1988).
 "Affymax raises \$25 million to develop high-speed drug discovery system," *Biotechnology News*, 10(3):7-8 (1990).
 "Preparation of fluorescent-labeled DNA and its use as a probe in molecular hybridization," *Bioorg Khim*, 12(11):1508-1513 (1986).
 Abbott et al., "Manipulation of the Wettability of Surfaces on the 0.1- to 1-Micrometer Scale Through Micromachining and Molecular Self-Assembly," *Science*, 257:1380-1382 (1992).
 Adams et al., "Complementary DNA Sequencing: Expressed Sequence Tags and Human Genome Project," *Science*, 252(5013):1651-1656 (1991).
 Adams et al., "Photolabile Chelators That 'Cage' Calcium with Improved Speed of Release and Pre-Photolysis Affinity," *J. Gen. Physiol.*, p. 9a (Dec. 1986).
 Adams et al., "Biologically Useful Chelators That Take Up Ca²⁺ upon Illumination," *J. Am. Chem. Soc.*, 111:7957-7968 (1989).
 Amit et al., "Photosensitive Protecting Groups of Amino Sugars and Their Use in Glycoside Synthesis. 2-Nitrobenzyloxycarbonylamino and 6-Nitroveratryloxycarbonylamino Derivatives," *J. Org. Chem.*, 39(2):192-196 (1974).
 Amit et al., "Photosensitive Protecting Groups—A Review," *Israel J. Chem.*, 12(1-2):103-113 (1974).
 Applied Biosystems, Model 431A Peptide Synthesizer User's manual, Sections 2 and 6, (Aug. 15, 1989).
 Ajayaghosh et al., "Solid-Phase Synthesis of N-Methyl- and N-Ethylamides of Peptides Using Photolytically Detachable ((3-Nitro-4((alkylamino)methyl)benzamido)methyl)polystyrene Resin," *J. Org. Chem.*, 55(9):2826-2829 (1990).
 Ajayaghosh et al., "Solid-phase synthesis of C-terminal peptide amides using a photoremovable α -methylphenacylamido anchoring linkage," *Proc. Ind. Natl. Sci (Chem.Sci.)*, 100(5):389-396 (1988).
 Ajayaghosh et al., "Polymer-supported Solid-phase Synthesis of C-Terminal Peptide N-Methylamides Using a Modified Photoremovable 3-Nitro-4-N-methylaminomethylpolystyrene Support," *Ind.J.Chem.*, 27B:1004-1008 (1988).
 Ajayaghosh et al., "Polymer-Supported Synthesis of Protected Peptide Segments on a Photosensitive o-Nitro(α -Methyl)Bromobenzyl Resin," *Tetrahedron*, 44(21):6661-6666 (1988).
 Arnold et al., "A Novel Universal Support for DNA & RNA Synthesis," abstract from Federation Proceedings, 43(7):abstract No. 3669 (1984).
 Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, (1989), tbl of cont., pp. vii-ix.
 Augenlicht et al., "Cloning and Screening of Sequences Expressed in a Mouse Colon Tumor," *Cancer Research*, 42:1088-1093 (1982).

- Augenlicht et al., "Expression of Cloned Sequences in Biopsies of Human Colonic Tissue and in Colonic Carcinoma Cells Induced to Differentiate in Vitro," *Cancer Res.*, 47:6017-6021 (1987).
- Bains, W., "Hybridization Methods for DNA Sequencing," *Genomics*, 11(2):294-301 (1991).
- Bains et al., "A Novel Method for Nucleic Acid Sequence Determination," *J. Theor. Biol.*, 135:303-307 (1988).
- Bains, W., "Alternative Routes Through the Genome," *Bio-technology*, 8:1251-1256 (1990).
- Balachander et al., "Functionalized Siloxy-Anchored Monolayers with Exposed Amino, Azido, Bromo, or Cyano Groups," *Tetrahed. Lett.*, 29(44):5593-5594 (1988).
- Baldwin et al., "New Photolabile Phosphate Protecting Groups," *Tetrahed.*, 46(19):6879-6884 (1990).
- Barltrop et al., "Photosensitive Protective Groups," *Chemical Communications*, pp. 822-823 (1966).
- Barinaga, M., "Will 'DNA Chip' Speed Genome Initiative," *Science*, 253:1489 (1985).
- Bart et al., "Microfabricated Electrohydrodynamic Pumps," *Sensors and Actuators*, A21-A23:193-197 (1990).
- Bartsh et al., "Cloning of mRNA sequences from the human colon: Preliminary characterisation of defined mRNAs in normal and neoplastic tissues," *Br.J.Can.*, 54:791-798 (1986).
- Baum, R., "Fledgling firm targets drug discovery process," *Chem. Eng. News*, pp. 10-11 (1990).
- Beltz et al., "Isolation of Multigene Families and Determination of Homologies by Filter Hybridization Methods," *Methods in Enzymology*, 100:266-285 (1983).
- Benschop, Chem. Abstracts 114(26):256643 (1991).
- Bhatia et al., "New Approach To Producing Patterned Biomolecular Assemblies," *J. American Chemical Society*, 114:4432-4433 (1992).
- Biorad Chromatography Electrophoresis Immunochemistry Molecular Biology HPLC catalog M 1987 p. 182.
- Blawas et al., "Step-and-Repeat Photopatterning of Protein Features Using Caged-Biotin-BSA: Characterization and Resolution," *Langmuir*, 14(15):4243-4250 (1998).
- Blawas, A.S., "Photopatterning of Protein Features using Caged-biotin-Bovine Serum Albumin," dissertation for Ph.D at Duke University in 1998.
- Bos et al., "Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia," *Nature*, 315:726-730 (1985).
- Boyle et al., "Differential distribution of long and short interspersed element sequences in the mouse genome: Chromosome karyotyping by fluorescence in situ hybridization," *PNAS*, 87:7757-7761 (1990).
- Brock et al., "Rapid fluorescence detection of in situ hybridization with biotinylated bovine herpesvirus-1 DNA probes," *J. Veterinary Diagnostic Invest.*, 1:34-38 (1989).
- Burgi et al., "Optimization in Sample Stacking for High-Performance Capillary Electrophoresis," *Anal. Chem.*, 63:2042-2047 (1991).
- Cameron et al., "Photogeneration of Organic Bases from o-Nitrobenzyl-Derived Carbamates," *J. Am. Chem. Soc.*, 113:4303-4313 (1991).
- Carrano et al., "A High-Resolution, Fluorescence-Based, Semiautomated Method for DNA Fingerprinting," *Genomics*, 4:129-136 (1989).
- Caruthers, M.H., "Gene Synthesis Machines: DNA Chemistry and Its Uses," *Science*, 230:281-285 (1985).
- Chatterjee et al., "Inducible Alkylation of DNA Using an Oligonucleotide-Quinone Conjugate," *Am. J. Chem. Soc.*, 112:6397-6399 (1990).
- Chee et al., "Accessing Genetic Information with High-Density DNA Arrays," *Science*, 274:610-614 (1996).
- Chehab et al., "Detection of sickle cell anaemia mutation by colour DNA amplification," *Lancet*, 335:15-17 (1990).
- Chehab et al., "Detection of specific DNA sequences by fluorescence amplification: A color complementation assay," *PNAS*, 86:9178-9182 (1989).
- Clevite Corp., Piezoelectric Technology, Data for Engineers.
- Corbett et al., "Reaction of Nitroso Aromatics with Glyoxylic Acid. A New Path to Hydroxamic Acids," *J. Org. Chem.*, 45:2834-2839 (1980).
- Craig et al., "Ordering of cosmid clones covering the Herpes simplex virus type 1 (HSV-1) genome: a test case for fingerprinting by hybridization," *Nuc. Acid. Res.*, 18(9):2653-2660 (1990).
- Cummings et al., "Photoactivable Fluorophores. I. Synthesis and Photoactivation of o-Nitrobenzyl-Quenched Fluorescent Carbamates," *Tetrahedron Letters*, 29(1):65-68 (1988).
- Diggelmann, "Investigating the VLSIPS synthesis process," Sep. 9, 1994.
- Di Mauro et al., "DNA Technology in Chip Construction," *Adv. Mater.*, 5(5):384-386 (1993).
- Drmanac et al., "Partial Sequencing by Oligo-Hybridization Concept and Applications in Genome Analysis," 1st Int. Conf. Electrophor., Supercomp., Hum. Genome pp. 60-74 (1990).
- Drmanac et al., "Sequencing by Oligonucleotide Hybridization: A Promising Framework in Decoding of the Genome Program?," 1st Int. Conf. Electrophor., Supercomp., Hum. Genome pp. 47-59 (1990).
- Drmanac et al., "Laboratory Methods, Reliable Hybridization of Oligonucleotides as Short as Six Nucleotides," *DNA and Cell Biol.*, 9(7):527-534 (1990).
- Drmanac et al., "Sequencing of Megabase Plus DNA by Hybridization: theory of the Method," *Genomics*, 4:114-128 (1989).
- Drmanac et al., "Sequencing of Megabase Plus DNA by Hybridization: Theory of the Method," abstract of presentation given at Cold Spring Harbor Symposium on Genome Mapping and Sequencing, Apr. 27, 1988 thru May 1, 1988.
- Dulcey et al., "Deep UV Photochemistry of Chemisorbed Monolayers: Patterned Coplanar Molecular Assemblies," *Science*, 252:551-554 (1991).
- Duncan et al., "Affinity Chromatography of a Sequence-Specific DNA Binding Protein Using Teflon-Linked Oligonucleotides," *Analytical Biochemistry*, 169:104-108 (1988).
- Effenhauser et al., "Glass Chips for High-speed Capillary Electrophoresis Separations with Submicrometer Plate Heights," *Anal. Chem.*, 65:2637-2642 (1993).
- Effenhauser et al., "High-Speed Separation of Antisense Oligonucleotides on a Micromachined Capillary Electrophoresis Device," *Anal. Chem.*, 66:2949-2953 (1994).
- Ekins et al., "High Specific Activity Chemiluminescent and Fluorescent Markers: their Potential Application to High Sensitivity and 'Multi-analyte' Immunoassays," *J. Bioluminescence Chemiluminescence*, 4:59-78 (1989).
- Ekins et al., "Development of Microspot Multi-Analyte Rationmetric Immunoassay Using dual Fluorescent-Labeled Antibodies," *Anal. Chimica Acta*, 227:73-96 (1989).

- Ekins et al., "Multianalyte Microspot Immunoassay-Microanalytical 'Compact Disk' of the Future," *Clin. Chem.*, 37(11):1955-1967 (1991).
- Ekins, R.P., "Multi-Analyte immunoassay*," *J. Pharmaceut. Biomedical Analysis*, 7(2):155-168 (1989).
- Evans et al., "Microfabrication for Automation of Molecular processes in Human Genome Analysis," *Clin. Chem.*, 41(11):1681 (1995).
- Evans et al., "Physical mapping of complex genomes by cosmid multiplex analysis," *PNAS*, 86:5030-5034 (1989).
- Ezaki et al., "Small-Scale DNA Preparation for Rapid Genetic Identification of *campylobacter* Species with Radioisotope," *Microbiol. Immunology*, 32(2):141-150 (1988).
- Fan et al., "Mapping small DNA sequences by fluorescence in situ hybridization directly on banded metaphase chromosomes," *PNAS*, 87(16):6223-6227 (1990).
- Fan et al., "Micromachining of Capillary Electrophoresis Injectors and Separators on Glass Chips and Evaluation of Flow at Capillary Intersections," *Anal. Chem.*, 66:177-184 (1994).
- Fettingner et al., "Stacked modules for micro flow systems in chemical analysis: concept and studies using an enlarged model," *Sensors and Actuators*, B17:19-25 (1993).
- Flanders et al., "A new interferometric alignment technique," *App. Phys. Ltrs.*, 31(7):426-429 (1977).
- Fodor et al., "Multiplexed biochemical assays with biological chips," *Nature*, 364:555-556 (1993).
- Fodor et al., "Light-directed, Spatially Addressable Parallel Chemical Synthesis," *Science*, 251:767-773 (1991).
- Forman et al., "Thermodynamics of Duplex Formation and Mismatch Discrimination on Photolithographically Synthesized Oligonucleotide Arrays," chapter 13 pp. 206-228 from *Molecular Modeling of Nucleic Acids*, ACS Symposium Series 682, Apr. 13-17, 1997, Leontis et al., eds.
- Frank et al., "Simultaneous Multiple Peptide Synthesis Under Continuous flow Conditions on Cellulose Paper Discs as Segmental Solid Supports," *Tetrahedron*, 44(19):6013-6040 (1988).
- Frank et al., "Automation of DNA Sequencing Reactions and Related Techniques: A Workstation for Micromanipulation of Liquids," *Bio/Technology*, 6:1211-1212 (1988).
- Frank et al., "Simultaneous Synthesis and Biological Applications of DNA Fragments: An Efficient and Complete Methodology," *Methods in Enzymology*, 154:221-250 (1987).
- Fuhr et al., "Travelling wave-driven microfabricated electrohydrodynamic pumps for liquids," *J. Micromech. Microeng.*, 4:217-226 (1994).
- Fuller et al., "Urethane-Protected Amino Acid N-Carboxy Anhydrides and Their Use in Peptide Synthesis," *J. Amer. Chem. Soc.*, 112(20):7414-7416 (1990).
- Furka et al., "General method for rapid synthesis of multi-component peptide mixtures," *Int. J. Peptide Protein Res.*, 37:487-493 (1991).
- Furka et al., "Cornucopia of Peptides by Synthesis," 14th Int. Congress of Biochem. abst.#FR:013, Jul. 10-15, 1988 Prague, Czechoslovakia.
- Furka et al., "More Peptides by Less Labour," abst. 288, Int. Symp. Med. Chem., Budapest Hungary Aug. 15-19, 1988.
- Gait, eds., pp. 1-115 from *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, (1984).
- Gazard et al., "Lithographic Technique Using Radiation-Induced Grafting of Acrylic Acid into Poly(Methyl Methacrylate) Films," *Polymer Engineering and Science*, 20(16):1069-1072 (1980).
- Gergen et al., "Filter replicas and permanent collections of recombinant DNA plasmids," *Nuc. Acids Res.*, 7(8):2115-2137 (1979).
- Getzoff et al., "Mechanisms of Antibody Binding to a Protein," *Science*, 235:1191-1196 (1987).
- Geysen et al., "Strategies for epitope analysis using peptide synthesis," *J. Immunol. Meth.*, 102:259-274 (1987).
- Geysen et al., "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid," *PNAS*, 81:3998-4002 (1984).
- Geysen et al., "A synthetic strategy for epitope mapping" from *Peptides: Chem. & Biol.*, Proc. of 10th Am. Peptide Symp., May 23-28, 1987, pp. 519-523, (1987).
- Geysen, "Antigen-antibody interactions at the molecular level: adventures in peptide synthesis," *Immunol. Today*, 6(12):364-369 (1985).
- Geysen et al., "Cognitive Features of Continuous Antigenic Determinants," from *Synthetic Peptides: Approaches to Biological Probes*, pp. 19-30, (1989).
- Geysen et al., "Chemistry of Antibody Binding to a Protein," *Science*, 235:1184-1190 (1987).
- Geysen et al., "The delineation of peptides able to mimic assembled epitopes," 1986 CIBA Symp., pp. 130-149.
- Geysen et al., "Cognitive Features of Continuous Antigenic Determinants," *Mol. Recognit.*, 1(1):1-10 (1988).
- Geysen et al., "A Prio Ri Delineation of a Peptide Which Mimics A Discontinuous Antigenic Determinant," *Mol. Immunol.*, 23(7):709-715 (1986).
- Gilon et al., "Backbone Cyclization: A New Method for Conferring Conformational Constraint on Peptides," *Biopolymers*, 31(6):745-750 (1991).
- Gineras et al., "Hybridization properties of immobilized nucleic acids," *Nuc. Acids Res.*, 15(13):5373-5390 (87).
- Gummerlock et al., "RAS Enzyme-Linked Immunoblot Assay Discriminates p21 Species: A Technique to Dissect Gene Family Expression," *Anal. Biochem.*, 180:158-168 (1989).
- Gurney et al., "Activation of a potassium current by rapid photochemically generated step increases of intracellular calcium in rat sympathetic neurons," *PNAS*, 84:3496-3500 (1987).
- Haase et al., "Detection of Two Viral Genomes in Single Cells by Double-Label Hybridization in Situ and Color Microradioautography," *Science*, 227:189-192 (1985).
- Hacia, et al., "Two color hybridization analysis using high density oligonucleotide arrays and energy transfer dyes," *Nuc. Acids Res.*, 26(16):3865-3866 (1998).
- Hack, M.L., "Conics Formed to Make Fluid & Industrial Gas Micromachines," *Genetic Engineering News*, 15(18):1, 29 (1995).
- Hagedorn et al., "Pumping of Water Solutions in Microfabricated Electrohydrodynamic Systems," from *Micro Electro Mechanical Systems conference in Travemunde Germany* (1992).
- Hanahan et al., "Plasmid Screening at High Colony Density," *Meth. Enzymology*, 100:333-342 (1983).
- Hanahan et al., "Plasmid screening at high colony density," *Gene*, 10:63-67 (1980).

- Haridasan et al., "Peptide Synthesis using Photolytically Cleavable 2-Nitrobenzyloxycarbonyl Protecting Group," *Proc. Indian Nat. Sci. Acad.*, 53A(6):717-728 (1987).
- Harrison et al., "Capillary Electrophoresis and Sample Injection Systems Integrated on a Planar Glass Chip," *Anal. Chem.*, 64:1926-1932 (1992).
- Harrison et al., "Micromachining a Minaturized Capillary Electrophoresis-Based Chemical Analysis System on a Chip," *Science*, 261:895-897 (1993).
- Harrison et al., "Towards minaturized electrophoresis and chemical analysis systems on silicon: an alternative to chemical sensors*," *Sensors and Actuators*, B10:107-116 (1993).
- Harrison et al., "Rapid separation of fluorescein derivatives using a micromachined capillary electrophoresis system," *Analytica Chimica Acta*, 283:361-366 (1993).
- Hellberg et al., "Minimum analogue peptide sets (MAPS) for quantitative structure-activity relationships," *Int. J. Peptide Protein Res.*, 37:414-424 (1991).
- Hilser et al., "Protein and peptide mobility in capillary zone electrophoresis, A comparison of existing models and further analysis," *J. Chromatography*, 630:329-336 (1993).
- Ho et al., "Highly Stable Biosensor Using an Artificial Enzyme," *Anal. Chem.*, 59:536-537 (1987).
- Hochgeschwender et al., "Preferential expression of a defined T-cell receptor β -chain gene in hapten-specific cytotoxic T-cell clones," *Nature*, 322:376-378 (1986).
- Hodgson, J., "Assays A La Photolithography," *Biotech.*, 9:419 (1991).
- Hopman et al., "Bi-color detection of two target DNAs by non-radioactive in situ hybridization*," *Histochem.*, 85:1-4 (1986).
- Iwamura et al., "1-Pyrenylmethyl Esters, Photolabile Protecting Groups for Carboxylic Acids," *Tetrahedron Lett.*, 28(6):679-682 (1987).
- Iwamura et al., "1-(α -Diazobenzyl)pyrene: A Reagent for Photolabile and Fluorescent Protection of Carboxyl Groups of Amino Acids and Peptides," *Synlett*, pp. 35-36 (1991).
- Jacobson et al., "Effects of Injection Schemes and Column Geometry on the Performance of Microchip Electrophoresis Devices," *Anal. Chem.*, 66:1107-1113 (1994).
- Jacobsen et al., "Open Channel Electrochromatography on a Microchip," *Anal. chem.*, 66:2369-2373 (1994).
- Jacobson et al., "Microchip Capillary Electrophoresis with an Integrated Postcolumn Reactor" *Anal. Chem.*, 66:3472-3476 (1994).
- Jacobson et al., "Precolumn Reactions with Electrophoretic Analysis Integrated on a Microchip," *Anal. Chem.*, 66:4127-4132 (1994).
- Jacobson et al., "Microfabricated chemical measurement systems," *Nature Medicine*, 1(10):1093-1096 (1995).
- Jacobsen et al., "Fused Quartz Substrates for Microchip Electrophoresis," *Anal. chem.*, 67:2059-2063 (1995).
- Jacobson et al., "High-Speed Separations on a Microchip," *Anal. Chem.*, 66:1114-1118 (1994).
- Jacobson et al., "Microchip electrophoresis with sample stacking," *Electrophoresis*, 16:481-486 (1995).
- Jayakumari, "Peptide synthesis in a triphasic medium catalysed by papain immobilized on a crosslinked polystyrene support," *Indian J. Chemistry*, 29B:514-517 (1990).
- Kaiser et al., "Peptide and Protein Synthesis by Segment Synthesis-Condensation," *Science*, 243:187-192 (1989).
- Kaplan et al., "Photolabile chelators for the rapid photorelease of divalent cations," *PNAS*, 85:6571-6575 (1988).
- Karube, "Micro-biosensors based on silicon fabrication technology," chapter 25 from *Biosensors: Fundamentals and Applications*, Turner et al., eds., Oxford Publ., 1987, pp. 471-480 (1987).
- Kates et al., "A Novel, Convenient, Three-dimensional Orthogonal Strategy for Solid-Phase Synthesis of Cyclic Peptides 1-3," *Tetrahed. Letters*, 34(10):1549-1552 (1993).
- Kerkof et al., "A Procedure for Making Simultaneous Determinations of the Relative Levels of Gene Transcripts in Tissues or Cells," *Anal. Biochem.*, 188:349-355 (1990).
- Khrapko et al., "An Oligonucleotide hybridization approach to DNA sequencing," *FEBS Lett.*, 256(1,2):118-122 (1989).
- Kievits et al., "Rapid subchromosomal localization of cosmid by nonradioactive in situ hybridization," *Cytogenetics Cell Genetics*, 53(2-3):134-136 (1990).
- Kimura et al., "An Immobilized Enzyme Membrane Fabrication Method using an Ink Jet Nozzle," *Biosensors*, 4:41-52 (1988).
- Kimura et al., "An Integrated SOF/FET Multi-Biosensor," *Sensors & Actuators*, 9:373-387 (1986).
- Kitazawa et al., "In situ DNA-RNA hybridization using in vivo bromodeoxyuridine-labeled DNA probe," *Histochemistry*, 92:195-199 (1989).
- Kleinfeld et al., "Controlled Outgrowth of Dissociated Neurons on Patterned Substrates," *J. Neurosci.*, 8(11):4098-4120 (1988).
- Knight, P., "Materials and Methods/Microsequencers for Proteins and Oligosaccharides," *Bio/Tech.*, 7:1075-76 (1989).
- Kohara et al., "The Physical Map of the Whole E. coli Chromosome: Application of a New Strategy for Rapid Analysis and Sorting of a Large Genomic Library," *Cell*, 50:495-508 (1987).
- Krile et al., "Multiplex holography with chirp-modulated binary phase-coded reference-beam masks," *Applied Opt.*, 18(1):52-56 (1979).
- Labat, I., "Subfragments as an informative characteristic of the DNA molecule—computer simulation," research report submitted to the University of Belgrade College of Nature Sciences and Mathematics, (1988).
- Lainer et al., "Human Lymphocyte Subpopulations Identified by Using Three-Color Immunofluorescence and Flow Cytometry Analysis: Correlation of Leu-2, Leu-3, Leu-7, Leu-8, and Leu-11 with Surface Antigen Expression," *Journal of Immunology*, 132(1):151-156 (1984).
- Lam et al., "A new type of synthetic peptide library for identifying ligand-binding activity," *Nature*, 354:82-84 (1991).
- Laskey et al., "Messenger RNA prevalence in sea urchin embryos measured with cloned cDNAs," *PNAS*, 77(9):5317-5321 (1980).
- Lee et al., "synthesis of a Polymer Surface Containing Covalently Attached Triethoxysilane Functionality: Adhesion to Glass," *Macromolecules*, 21:3353-3356 (1988).
- Allister et al., "Labelling oligonucleotides to high specific activity (I)," *Nuc. Acids Res.*, 17(12):4605-4610 (89).
- Frischauf et al., "Phage Vectors—EMBL Series," *Meth. Enzymology*, 153:103-115 (1987).
- Levy, M.F., "Preparing Additive Printed Circuits," *IBM Tech. Discl. Bull.*, 9(11):1473 (1967).
- Lichter et al., "High-Resolution Mapping of Human Chromosome 11 by in Situ hybridization with Cosmid Clones," *Science*, 247:64-69 (1988).

- Lichter et al., "Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines," *PNAS*, 87:6634-6638 (1990).
- Lichter et al., "Rapid detection of human chromosome 21 aberrations by in situ hybridization," *PNAS*, 85:9664-9668 (1988).
- Lichter et al., "Is non-isotopic in situ hybridization finally coming of age," *Nature*, 345:93-94 (1990).
- Lieberman et al., "A Light source Smaller Than the Optical Wavelength," *Science*, 247:59-61 (1990).
- Lipshutz et al., "Using Oligonucleotide Probe Arrays To Access Genetic Diversity," *BioTech.*, 19(3):442-7 (1995).
- Liu et al., "Sequential Injection Analysis in Capillary Format with an Electroosmotic Pump," *Talanta*, 41(11):1903-1910 (1994).
- Lockhart et al., "Expression monitoring by hybridization to high-density oligonucleotide arrays," *Nat. Biotech.*, 14:1675-1680 (1996).
- Logue et al., "General Approaches to Mask Design for Binary Optics," *SPIE*, 1052:19-24 (1989).
- Loken et al., "three-color Immunofluorescence Analysis of Leu Antigens on Human Peripheral Blood Using Two Lasers on a Fluorescence-Activated Cell Sorter," *Cytoetry*, 5:151-158 (1984).
- Love et al., "Screening of γ Library for Differentially Expressed Genes Using in Vitro Transcripts," *Anal. Biochem.*, 150:429-441 (1985).
- Lowe, C.R., "Biosensors," *Trends in Biotech.*, 2:59-65 (1984).
- Lowe, C.R., "An Introduction to the Concepts and Technology of Biosensors," *Biosensors*, 1:3-16 (1985).
- Lowe, C.R., *Biotechnology and Crop Improvement and Protection*, BCPC Publications, pp. 131-138 (1986).
- Lowe et al., "Solid-Phase Optoelectronic Biosensors," *Methods in Enzymology*, 137:338-347 (1988).
- Lowe, C.R., "Biosensors," *Phil. Tran. R. Soc. Lond.*, 324:487-496 (1989).
- Lu et al., "Differential screening of murine ascites cDNA libraries by means of in vitro transcripts of cell-cycle-phase-specific cDNA and digital image processing," *Gene*, 86:185-192 (1990).
- Lysov et al., "A new method for determining the DNA nucleotide sequence by hybridization with oligonucleotides," *Doklady Biochem.*, 303(1-6):436-438 (1989).
- Lysov et al., "DNA Sequencing by Oligonucleotide Hybridization," First International Conference on Electrophoresis, Supercomputing and the Human Genome, Apr. 10-13, 1990 p. 157.
- MacDonald et al., "A Rapid ELISA for Measuring Insulin in a Large Number of Research Samples," *Metabolism*, 38(5):450-452 (1989).
- Manz et al., "Miniaturized Total Chemical Analysis Systems: a Novel Concept for Chemical Sensing," *Sensors and Actuators*, B1:244-248 (1990).
- Manz et al., "Micromachining of monocrystalline silicon and glass for chemical analysis systems, A look into next century's technology or just a fashionable craze?," *Trends in Analytical Chem.*, 10(5):144-149 (1991).
- Manz et al., "Planar chips technology for miniaturization and integration of separation techniques into monitoring systems, Capillary electrophoresis on a chip," *J. Chromatography*, 593:253-258 (1992).
- Manz et al., "Planar Chips Technology for Miniaturization of Separation Systems: A Developing Perspective in Chemical Monitoring," chapter 1, 1-64 (1993).
- Manz et al., "Electroosmotic pumping and electrophoretic separations for miniaturized chemical analysis systems," *J. Micromech. Microeng.*, 4:257-265 (1994).
- Masiakowski et al., "Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line," *Nuc. Acids Res.*, 10(24):7895-7903 (1982).
- Matsumoto et al., "Preliminary Investigation of Micropumping Based on Electrical Control of Interfacial Tension," *IEEE*, pp. 105-110 (1990).
- Matsuzawa et al., "Containment and growth of neuroblastoma cells on chemically patterned substrates," *J. Neurosci. Meth.*, 50:253-260 (1993).
- McCray et al., "Properties and Uses of Photoreactive Caged Compounds," *Ann. Rev. Biophys. Biophys. Chem.*, 18:239-270 (1989).
- McGall et al., "The Efficiency of Light-Directed Synthesis of DNA Arrays on Glass Substrates," *J. American Chem. Soc.*, 119(22):5081-5090 (1997).
- McGillis, VLSI Technology, Sze, eds., Chapter 7, "Lithography," pp. 267-301 (1983).
- McMurray, J.S., "Solid Phase Synthesis of a Cyclic Peptide Using Fmoc Chemistry," *Tetrahedron Letters*, 32(52):7679-7682 (1991).
- Meinkoth et al., "Review: Hybridization of Nucleic Acids Immobilized on solid Supports," *Analytical Biochem.*, 138:267-284 (1984).
- Melcher et al., "Traveling-Wave Bulk Electroconvection Induced across a Temperature Gradient," *Physics of Fluids*, 10(6):1178-1185 (1967).
- Merrifield, R.B., "Solid Phase peptide Synthesis. I. The Synthesis of a Tetrapeptide," *J. Am. Chem. Soc.*, 85:2149-2154 (1963).
- Michiels et al., "Molecular approaches to genome analysis: a strategy for the construction of ordered overlapping clone libraries," *CABIOS*, 3(3):203-10 (1987).
- Mirzabekov, A.D., "DNA sequencing by hybridization—a megasequencing method and a diagnostic tool?," *TIBTECH*, 12:27-32 (1994).
- Monaco et al., "Human Genome Linking with Cosmids and Yeast Artificial Chromosomes," abstract from CSHS, p. 50, (1989).
- Morrison et al., "Solution-Phase Detection of Polynucleotides Using Interacting Fluorescent Labels and Competitive Hybridization," *Anal. Biochem.*, 183:231-244 (1989).
- Mutter et al., "Impact of Conformation on the Synthetic Strategies for Peptide Sequences," pp. 217-228 from *Chemistry of Peptides and Proteins*, vol. 1, Proceedings of the Third USSR-FRG Symp., in USSR (1982).
- Nakamori et al., "A Simple and Useful Method for Simultaneous Screening of Elevated Levels of Expression of a Variety of Oncogenes in Malignant Cells," *Jpn. J. Cancer Res.*, 79:1311-1317 (1988).
- Nederlof et al., "Multiple Fluorescence In Situ Hybridization," *Cytometry*, 11:126-131 (1990).
- Nyborg, W., "Acoustic Streaming," chapter 11 pp. 265-329 from *Physical Acoustics, Principles and Methods*, Mason, eds., vol. II, part B, Academic Press, New York and London (1965).
- Ocvirk et al., "High Performance Liquid Chromatography Partially Integrated onto a Silicon Chip," *Analyt. Meth. Instrumentation*, 2(2):74-82 (1995).

- Ohtsuka et al., "Studies on transfer ribonucleic acids and related compounds. IX Ribonucleic oligonucleotide synthesis using a photosensitive 0-nitrobenzyl protection at the 2'-hydroxyl group," *Nuc.Acids.Res.*, 1(10):1351-1357 (1974).
- Olefirowicz et al., "Capillary Electrophoresis for Sampling Single Nerve Cells," *Chimia*, 45(4):106-108 (1991).
- Patchornik et al., "Photosensitive Protecting Groups," *J.Am.Chem.Soc.*, 92(21):6333-6335 (1970).
- Patent Abstracts of Japan from EPO, Abst. 13:557, JP 1-233 447 (1989).
- Pease et al., "Light-generated oligonucleotide arrays for rapid DNA sequence analysis," *PNAS*, 91:5022-26 (1994).
- Pevzner, P.A., "1-Tuple DNA Sequencing: Computer Analysis," *J. Biomol. Struct. Dynam.*, 7(1):63-69 (1989).
- Pfahler et al., "Liquid Transport in Micron and Submicron Channels," *Sensors and Actuators*, A21-A23:431-4 (90).
- Pidgeon et al., "Immobilized Artificial Membrane Chromatography: Supports Composed of Membrane Lipids," *Anal. Biochem.*, 176:36-47 (89).
- Pillai, V.N., "Photoremovable Protecting Groups in Organic Synthesis," *Synthesis*, pp. 1-26 (1980).
- Pillai et al., "3-Nitro-4-Aminomethylbenzoyl derivative von Polyethylenglykolen: Eine neue Klasse von Photosensitiven löslichen Polymeren Trägern zur Synthese von C-terminalen Peptidamiden," *Tetrah. Ltr.*, # 36 pp. 3409-3412 (1979).
- Pillai et al., "Synthetic Hydrophilic Polymers, Biomedical and Chemical Applications," *Naturwissenschaften*, 68:558-566 (1981).
- Pirrung et al., "Proofing of Photolithographic DNA Synthesis with 3',5'-Dimethoxybenzoinyloxycarbonyl-Protected Deoxynucleoside Phosphoramidites," *J. Org. Chem.*, 63(2):241-246 (1998).
- Pirrung et al., "Comparison of Methods for Photochemical Phosphoramidite-Based DNA Synthesis," *J. Org. Chem.*, 60:6270-6276 (1995).
- Ploax et al., "Cyclization of peptides on a solid support," *Int. J. Peptide Protein Research*, 29:162-169 (1987).
- Polsky-Cynkin et al., "Use of DNA Immobilized on Plastic and Agarose Supports to Detect DNA by Sandwich Hybridization," *Clin. Chem.*, 31(9):1428-1443 (1985).
- Poustka et al., "Molecular Approaches to Mammalian Genetics," Cold Spring Harbor Symposia on Quantitative Biology, 51:131-139 (1986).
- Purushothaman et al., "Synthesis of 4,5-diarylimidazole-2-thiones and their photoconversion to bis(4,5-diarylimidazole-2-yl) sulphides," *Ind. J. Chem.*, 29B:18-21 (1990).
- Quesada et al., "High-Sensitivity DNA Detection with a Laser-Exited Confocal Fluorescence Gel Scanner," *Biotechniques*, 10:616 (1991).
- Reichmanis et al., *J. Polymer Sci. Polymer Chem. Edition*, 23:1-8 (1985).
- Richter et al., "An Electrohydrodynamic Micropump," *IEEE*, pp. 99-104 (1990).
- Richter et al., "Electrohydrodynamic Pumping and Flow Measurement," *IEEE*, pp. 271-276 (1991).
- Richter et al., "A Micromachined electrohydrodynamic (EHD) pump," *Sensors and Actuators*, A29:159-168 (91).
- Robertson et al., "A General and Efficient Route for Chemical Aminoacylation of Transfer RNAs," *J. Am. Chem. Soc.*, 113:2722-2729 (1991).
- Rodda et al., "The Antibody Response to Myoglobin-I. Systematic Synthesis of Myoglobin Peptides Reveals Location and Substructure of Species-Dependent Continuous Antigenic Determinants," *Mol. Immunol.*, 23(6):603-610 (1986).
- Rodgers, R.P., "Data Processing of Immunoassay Results," *Manual of Clin. Lab. Immunol.*, 3rd ed., ch. 15, pp. 82-87 (1986).
- Rose, D.J., "Free-solution reactor for post-column fluorescence detection in capillary zone electrophoresis," *J. Chromatography*, 540:343-353 (1991).
- Rovero et al., "Synthesis of Cyclic Peptides on solid Support," *Tetrahed. Letters*, 32(23):2639-2642 (1991).
- Saiki et al., "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes," *PNAS*, 86:6230-6234 (1989).
- Saiki et al., "Analysis of enzymatically amplified β -globin and HLA-DQ α DNA with Allele-specific oligonucleotide probes," *Nature*, 324:163-166 (1986).
- Scharf et al., "HLA class II allelic variation and susceptibility to pemphigus vulgaris," *PNAS*, 85(10):3504-3508 (1988).
- Schuup et al., "Mechanistic Studies of the Photorearrangement of o-Nitrobenzyl Esters," *J. Photochem.*, 36:85-97 (1987).
- Seiler et al., "Planar Glass Chips for Capillary Electrophoresis: Repetitive Sample Injection, Quantitation, and Separation Efficiency," *Anal. Chem.*, 65:1481-1488 (1993).
- Seller et al., "Electroosmotic Pumping and Valveless Control of Fluid Flow within a Manifold of Capillaries on a Glass Chip," *Anal. Chem.*, 66:3485-3491 (1994).
- Sheldon et al., "Matrix DNA Hybridization," *Clinical Chemistry*, 39(4):718-719 (1993).
- Shin et al., "Dehydrooligonopeptides. XI. Facile Synthesis of Various Kinds of Dehydrodi- and tripeptides, and Dehydroenkephalins Containing Tyr Residue by Using N-Carboxydehydrotyrosine Anhydride," *Bull. Chem. Soc. Jpn.*, 62:1127-1135 (1989).
- Sim et al., "Use of a cDNA Library for Studies on Evolution and Development Expression of the Chorion Multigene Families," *Cell*, 18:1303-1316 (1979).
- Smith et al., "A Novel Method for Delineating Antigenic Determinants: Peptide Synthesis and Radioimmunoassay Using the Same Solid Support," *Immunochemistry*, 14:565-568 (1977).
- Southern et al., "Report on the Sequencing by Hybridization Workshop," *Genomics*, 13:1378-1383 (1992).
- Southern et al., "Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesized in situ," *Nuc. Acids Res.*, 20(7):1679-1684 (1992).
- Southern et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation Using Experimental Models," *Genomics*, 13:1008-10017 (1992).
- Stemme et al., "A valveless diffuser/nozzle-based fluid pump," *Sensors and Actuators*, A39:159-167 (1993).
- Stryer, L., "DNA Probes and Genes Can be Synthesized by Automated Solid-Phase Methods," from *Biochemistry*, Third Edition, published by W.H. Freeman & Co., (1988).
- Stuber et al., "Synthesis and photolytic cleavage of bovine insulin B22-30 on a nitrobenzoylglycyl-poly (ethylene glycol) support," *Int. J. Peptide Protein Res.*, 22(3):277-283 (1984).

- Sundberg et al., "Spatially-Addressable Immobilization of Macromolecules on Solid Supports," *J. Am. Chem. Soc.*, 117(49):12050-12057 (1995).
- Swedberg, S.A., "Use of non-ionic and zwitterionic surfactants to enhance selectivity in high-performance capillary electrophoresis, An apparant micellar electrokinetic capillary chromatography mechanism," *J. Chromatography*, 503:449-452 (1990).
- Titus et al., "Texas Red, a Hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies," *J. Immunol. Meth.*, 50:193-204 (1982).
- Tkachuk et al., "Detection of bcr-abl Fusion in chronic Myelogenous Leukemia by in situ Hybridization," *Science*, 250:559-562 (90).
- Trzeciak et al., "Synthesis of 'Head-to-Tail' Cyclized Peptides on Solid Support by Fmoc Chemistry," *Tetrahed. Letters*, 33(32):4557-4560 (1992).
- Tsien et al., "Control of Cytoplasmic Calcium with Photolabile Tetracarboxylate 2-Nitrobenzhydryl Chelators," *Biophys. J.*, 50:843-853 (1986).
- Tsutsumi et al., "Expression of L- and M-Type Pyruvate Kinase in Human Tissues," *Genomics*, 2:86-89 (1988).
- Turchinskii et al., "Multiple Hybridization in Genome Analysis, Reaction of Diamines and Bisulfate with Cytosine for Introduction of Nonradioactive labels Into DNA," *Molecular Biology*, 22:1229-1235 (1988).
- Turner et al., "Photochemical Activation of Acylated α -Thrombin," *J. Am. Chem. Soc.*, 109:1274-1275 (1987).
- Urdea et al., "A novel method for the rapid detection of specific nucleotide sequences in crude biological sample without blotting or radioactivity; application to the analysis of hepatitis B virus in human serum," *Gene*, 61:253-264 (1987).
- Urdea et al., "A comparison of non-radioisotope hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes," *Nuc. Acids Res.*, 16(11):4937-4956 (1988).
- Van der Voort et al., "Design and Use of a Computer Controlled Confocal Microscopic for Biological Applications," *Scanning*, 7(2):66-78 (1985).
- Van Hijfte et al., "Intramolecular 1,3-Diyl Trapping Reactions. A Formal Total Synthesis of -Coriolin," *J. Organic Chemistry*, 50:3942-3944 (1985).
- Veldkamp, W.B., "Binary optics: the optics technology of the 1990s," *CLEO 90*, vol. 7, paper # CMG6 (1990).
- Verlaan-de Vries et al., "A dot-blot screening procedure for mutated ras oncogenes using synthetic oligodeoxynucleotides," *Gene*, 50:313-320 (1986).
- Verpoorte et al., "Three-dimensional micro flow manifolds for miniaturized chemical analysis systems," *J. Micromech. Microeng.*, 4:246-256 (1994).
- Volkmut et al., "DNA electrophoresis in microlithographic arrays," *Nature*, 358:600-602 (1992).
- Voss et al., "The immobilization of oligonucleotides and their hybridization properties," *Biochem. Soc. Transact.*, 16:216-217 (1988).
- Walker et al., "Photolabile Protecting Groups for an Acetylcholine Receptor Ligand. Synthesis and Photochemistry of a New Class of o-Nitrobenzyl Derivatives and their Effects on Receptor Function," *Biochemistry*, 25:1799-1805 (1986).
- Wallace et al., "Hybridization of synthetic oligodeoxyribonucleotides to $\Phi\chi$ 174 DNA: the effect of single base pair mismatch," *Nuc. Acids Res.*, 11(6):3543-3557 (1979).
- Washizu et al., "Handling Biological Cells Using a Fluid Integrated Circuit," *IEEE Transactions Industry Applications*, 26(2):352-358 (1990).
- Werner et al., "Size-Dependent Separation of Proteins Denatured in SDS by Capillary Electrophoresis Using a Replaceable Sieving Matrix," *Anal. Biochem.*, 212:253-258 (1993).
- White et al., "An Evaluation of Confocal Versus Conventional Imaging of Biological Structures by Fluorescence Light Microscopy," *J. Cell Biol.*, 105(1):41-48 (1987).
- Widacki et al., "Biochemical Differences in Qa-2 Antigens Expressed by Qa-2+,6+ and Qa-2a+,6- Strains. Evidence for Differential Expression of the Q7 and Q9 Genes," *Mol. Immunology*, 27(6):559-570 (1990).
- Wilcox et al., "Synthesis of Photolabile 'Precursors' of Amino Acid Neurotransmitters," *J. Org. Chem.*, 55:1585-1589 (1990).
- Wilding et al., "PCR in a Silicon Microstructure," *Clin. Chem.*, 40(9):1815-1818 (1994).
- Wilding et al., "Manipulation and Flow of Biological Fluids in Straight Channels Micromachined in Silicon," *Clin. Chem.*, 40(1):43-47 (1994).
- Wittman-Liebold, eds., *Methods in Protein Sequence Analysis*, from Proceedings of 7th Int'l Conf., Berlin, Germany, Jul. 3-8, 1988, table of contents, pp. xi-xx* (1989).
- Woolley et al., "Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips," *PNAS*, 91:11348-11352 (1994).
- Wu et al., "Synthesis and Properties of Adenosine-5'-triphospho- γ -5-(5-sulfonic acid)naphthyl Ethylamide: A Fluorescent Nucleotide Substrate for DNA-Dependent RNA Polymerase from *Escherichia coli*," *Arch. Biochem. Biophys.*, 246(2):564-571 (1986).
- Wu et al., "Laboratory Methods, Direct Analysis of Single Nucleotide Variation in Human DNA and RNA Using In Situ Dot Hybridization," *DNA*, 8(2):135-142 (1989).
- Yamamoto et al., "Features and applications of the laser scanning microscope," *J. Mod. Optics*, 37(11):1691-1701 (1990).
- Yarbrough et al., "Synthesis and Properties of Fluorescent Nucleotide Substrates for DNA-dependent RNA Polymerases," *J. Biol. Chem.*, 254(23):12069-12073 (1979).
- Yosomiya et al., "Performance, Glass fiber Having Isocyanate Group on the Surface. Preparation and Reaction with Amino Acid," *Polymer Bulletin*, 12:41-48 (1984).
- Young, W.S., "Simultaneous Use of Digoxigenin- and Radiolabeled Oligodeoxyribonucleotide Probes for Hybridization Histochemistry," *Neuropeptides*, 13:271-275 (1989).
- Yue et al., "Miniature Field-Flow Fractionation System for Analysis of Blood Cells," *Clin. Chem.*, 40(9):1810-1814 (1994).
- Zehavi et al., "Light-Sensitive Glycosides. I. 6-Nitroveratryl β -D-Glucopyranoside and 2-Nitrobenzyl β -D-Glucopyranoside," *J. Org. Chem.*, 37(14):2281-2285 (1972).
- Zengerle et al., "Transient measurements on miniaturized diaphragm pumps in microfluid systems," *Sensors and Actuators*, A46-47:557-561 (1995).

Ekins et al., "Fluorescence Spectroscopy and its Application to a New Generation of High Sensitivity, Multi-Microspot, Multianalyte, Immunoassay," *Clin. Chim. Acta*, 194:91-114 (1990).

Hames et al., *Nuclear acid hybridization, a practical approach*, cover page and table of contents (1985).

Mairanovsky, V. G., "Electro-Deprotection-Electrochemical Removal of Protecting Groups**," *Angew. Chem. Int. Ed. Engl.*, 15(5):281-292 (1976).

Morita et al., "Direct pattern fabrication on silicone resin by vapor phase electron beam polymerization," *J. Vac. Sci. Technol.*, B1(4):1171-1173 (1983).

Munegumi et al., "thermal Synthesis of Polypeptides from N-Boc-Amino Acid (Aspartic Acid, β -Aminoglutaric Acid) Anhydrides," *Chem. Letters*, pp. 1643-1646 (1988).

Sambrook, *Molecular Cloning - A Laboratory Manual*, publ. in 1989 (not included).

Semmelhack et al., "Selective Removal of Protecting Groups Using Controlled Potential Electrolysis," *J. Am. Chem. Society*, 94(14):5139-5140 (1972).

* cited by examiner

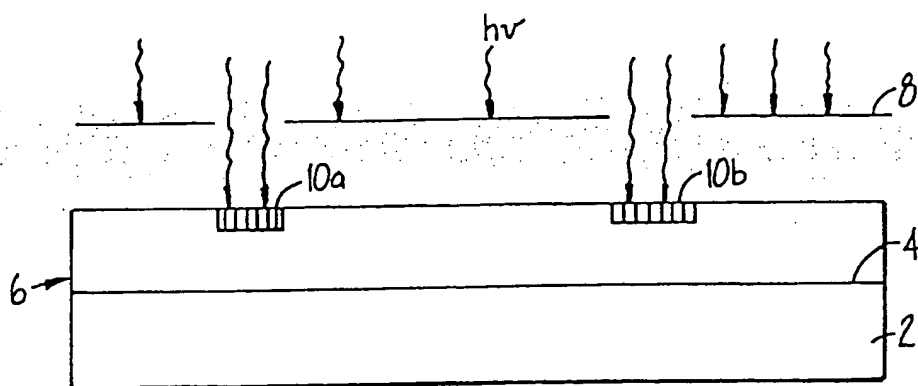


FIG. 1.

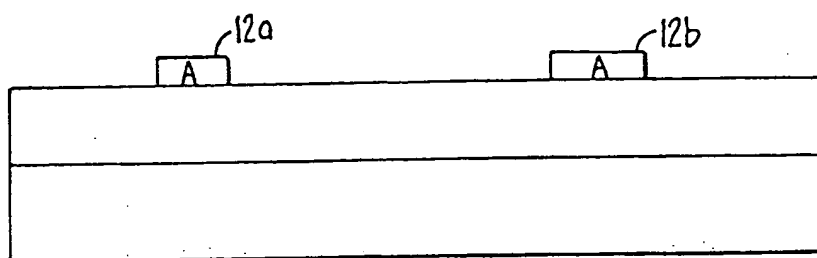


FIG. 2.

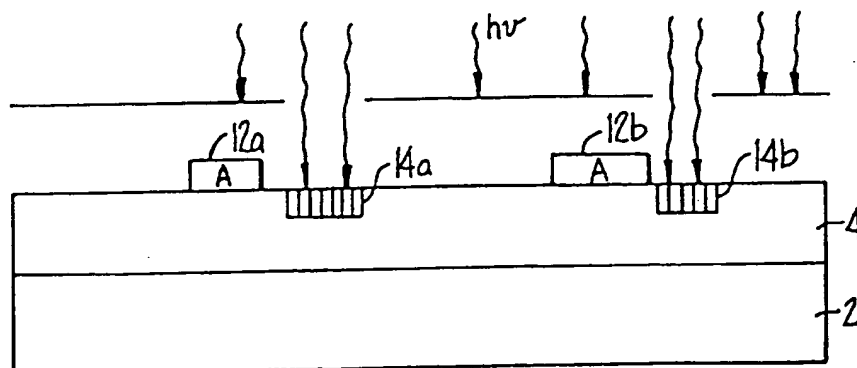


FIG. 3.

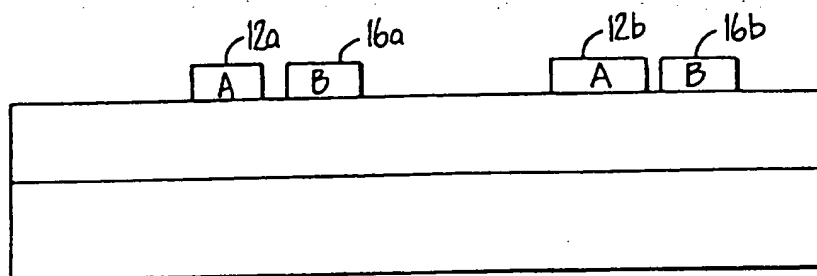


FIG. 4.

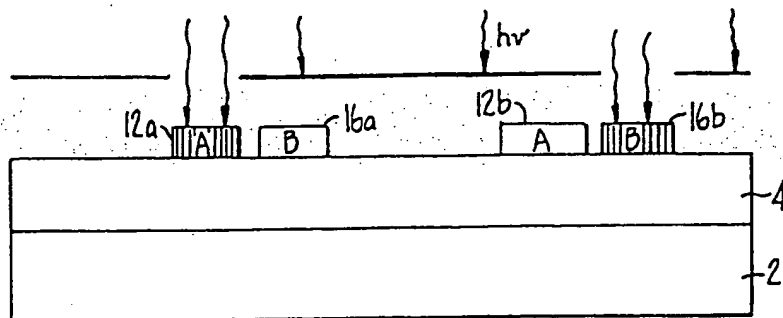


FIG. 5.

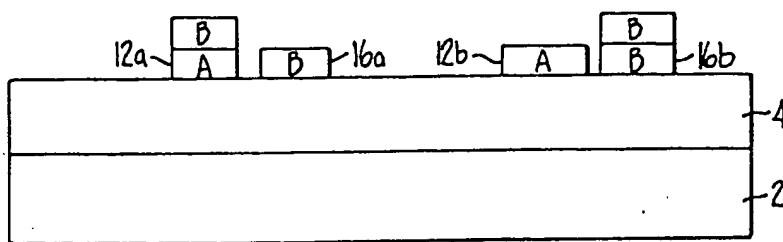


FIG. 6.

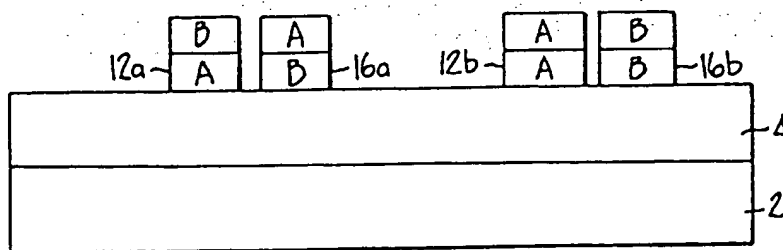


FIG. 7.

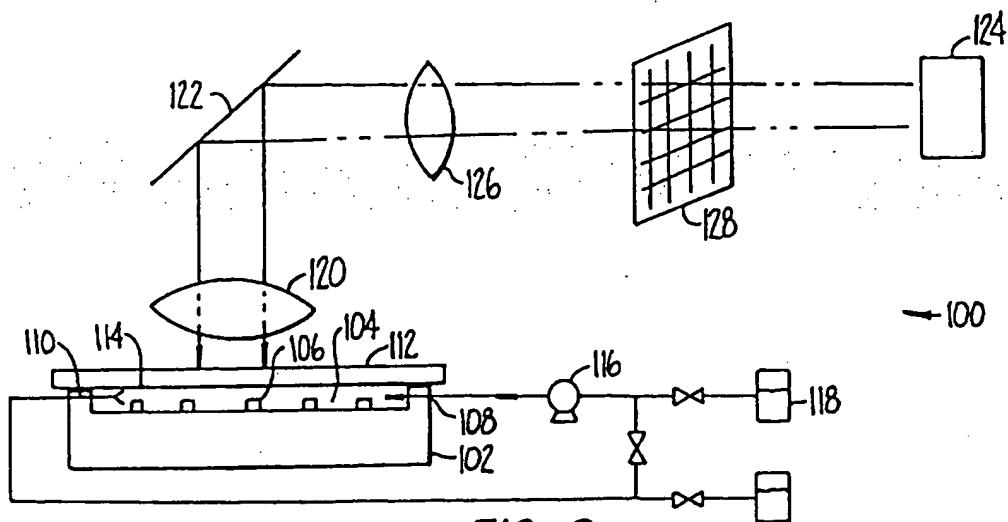


FIG. 8a.

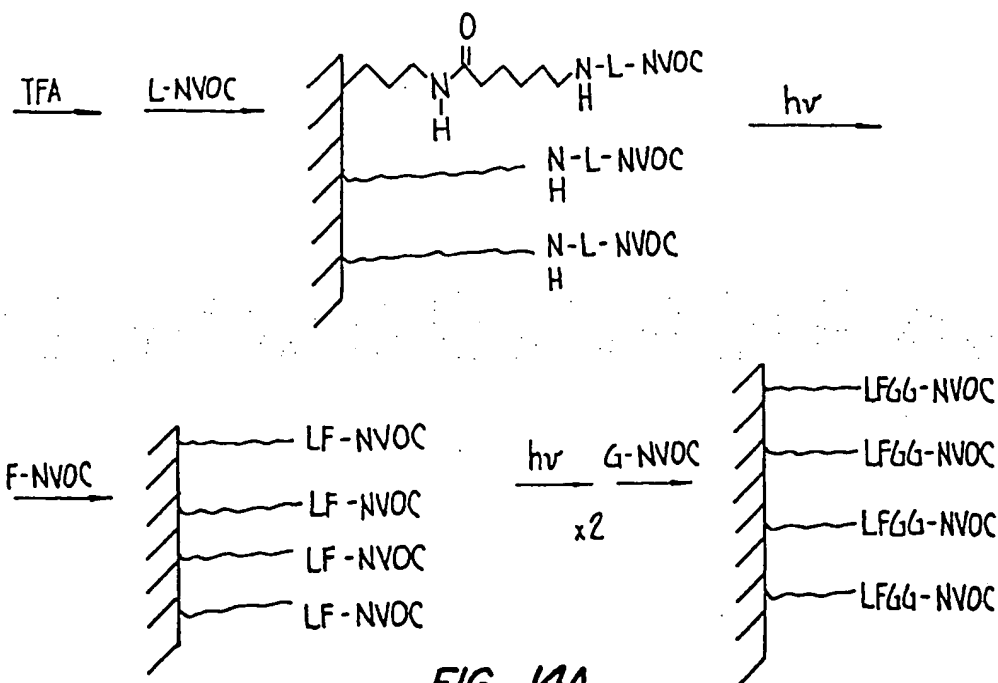
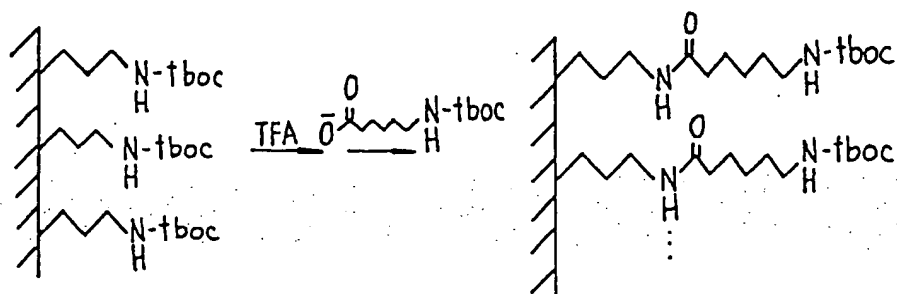
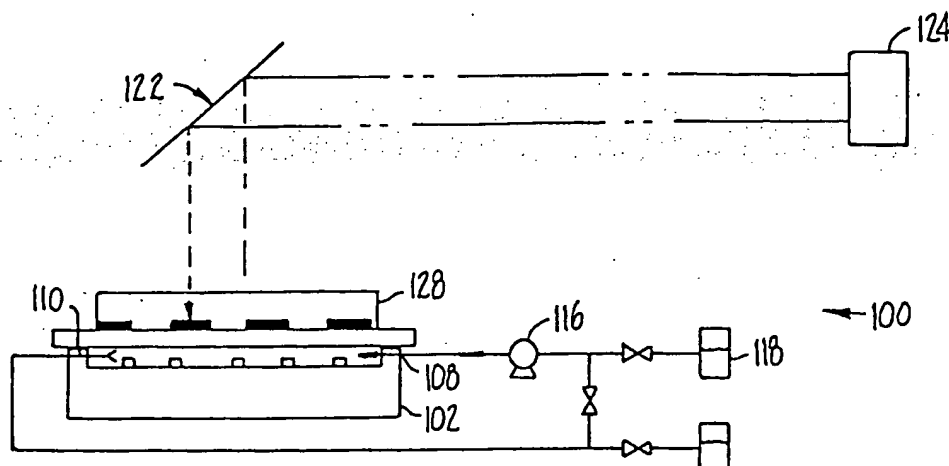


FIG. 14A.

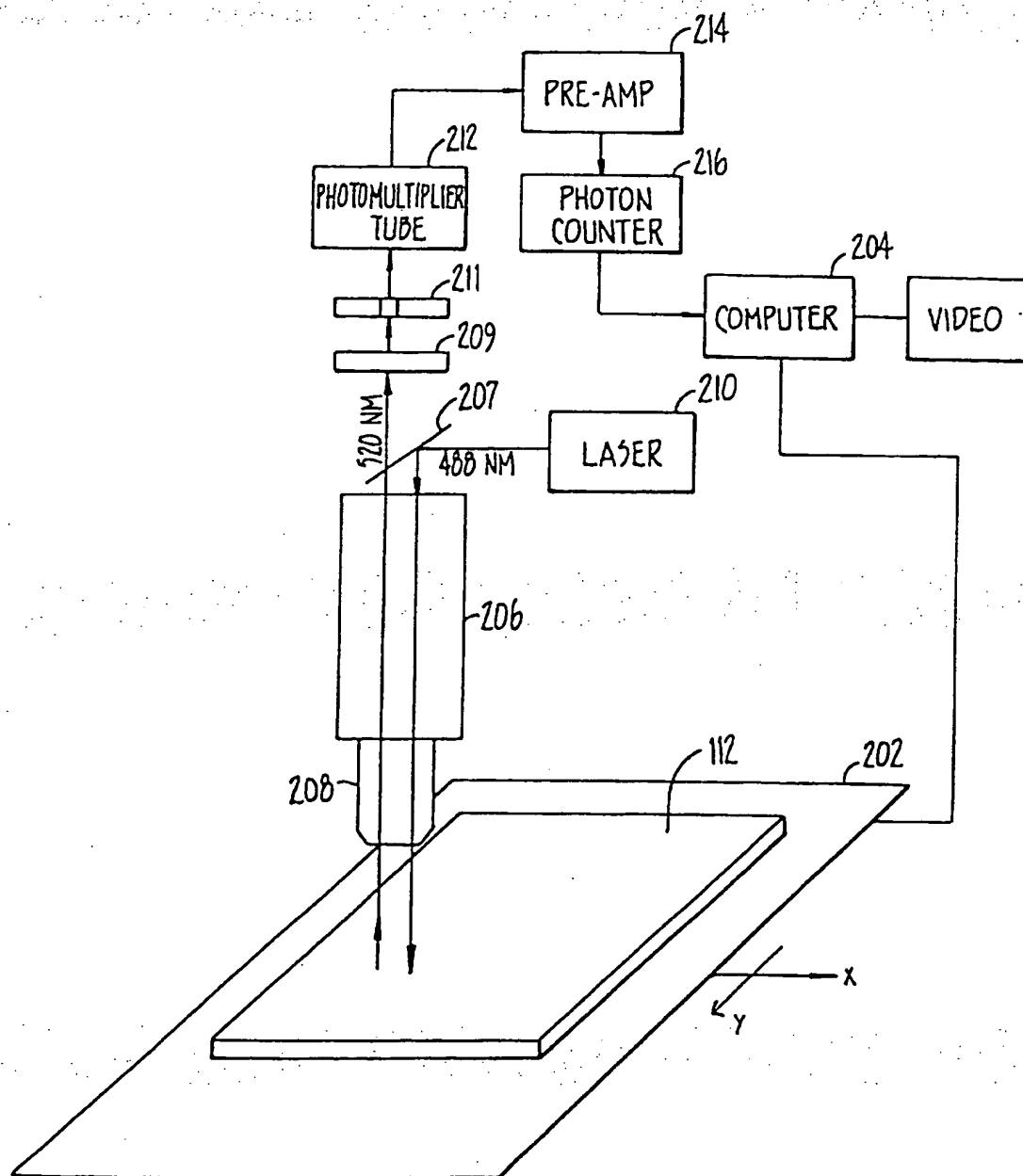


FIG. 9.

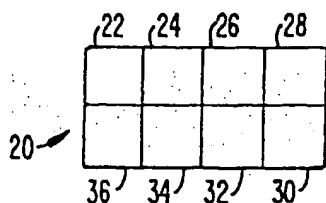


FIG. 10A.

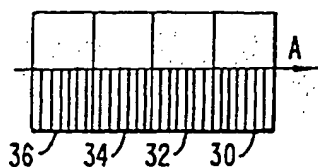


FIG. 10B.

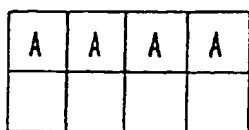


FIG. 10C.

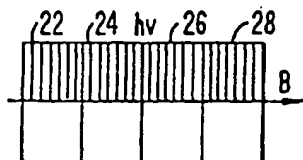


FIG. 10D.

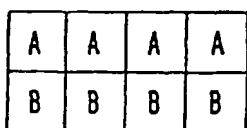


FIG. 10E.

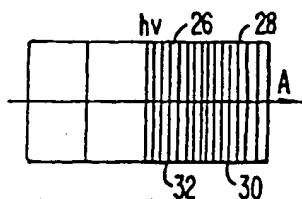


FIG. 10F.

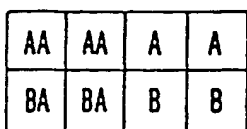


FIG. 10G.

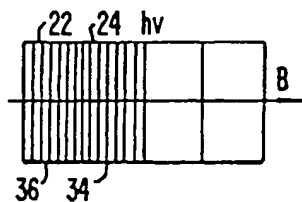


FIG. 10H.

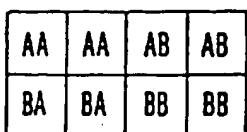


FIG. 10I.

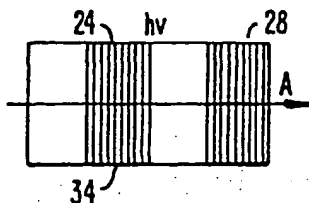


FIG. 10J.

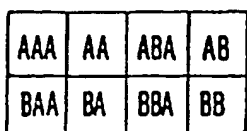


FIG. 10K.

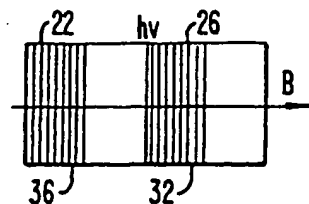


FIG. 10L.

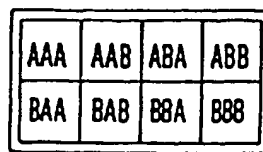


FIG. 10M.

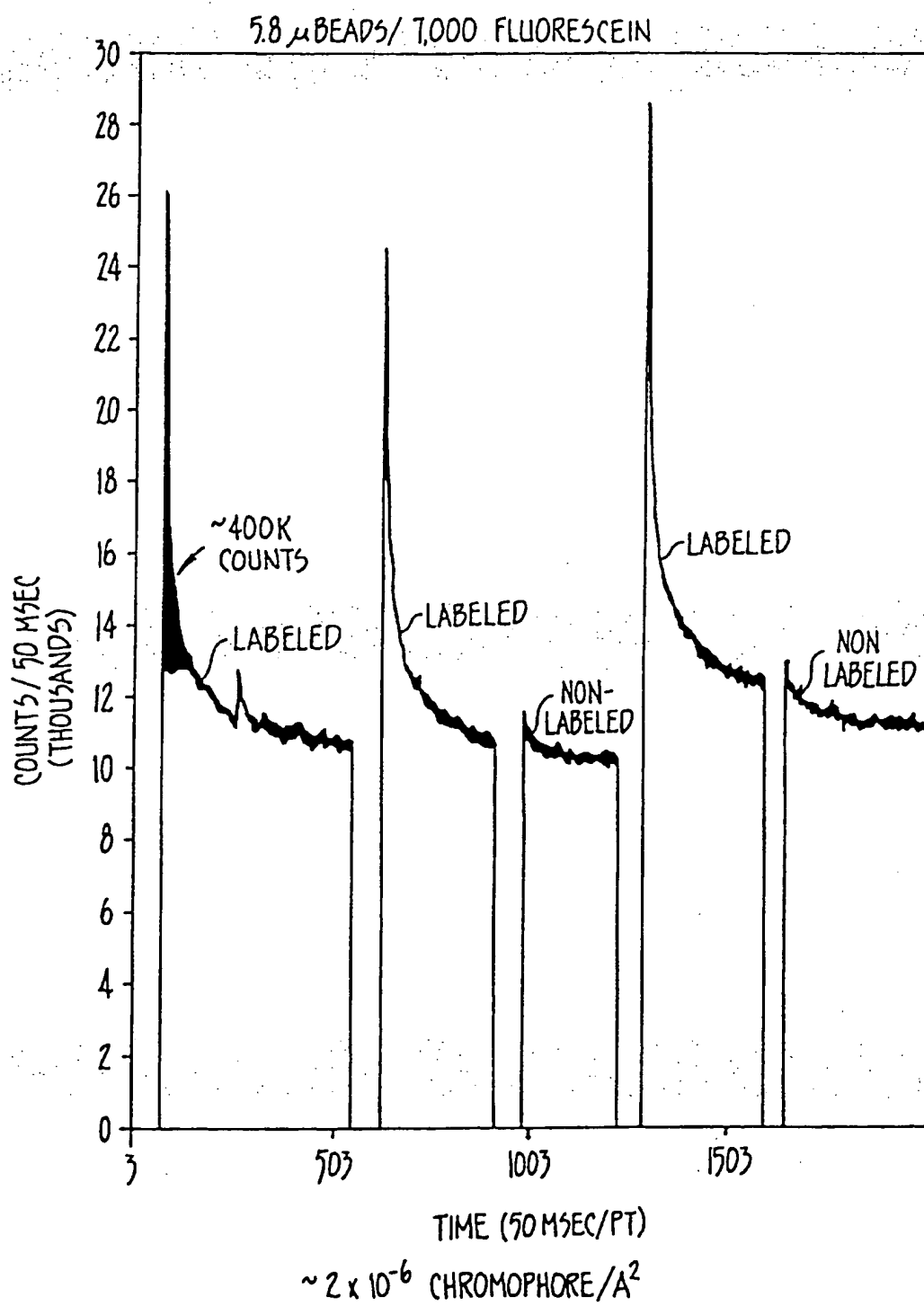


FIG. IIA.

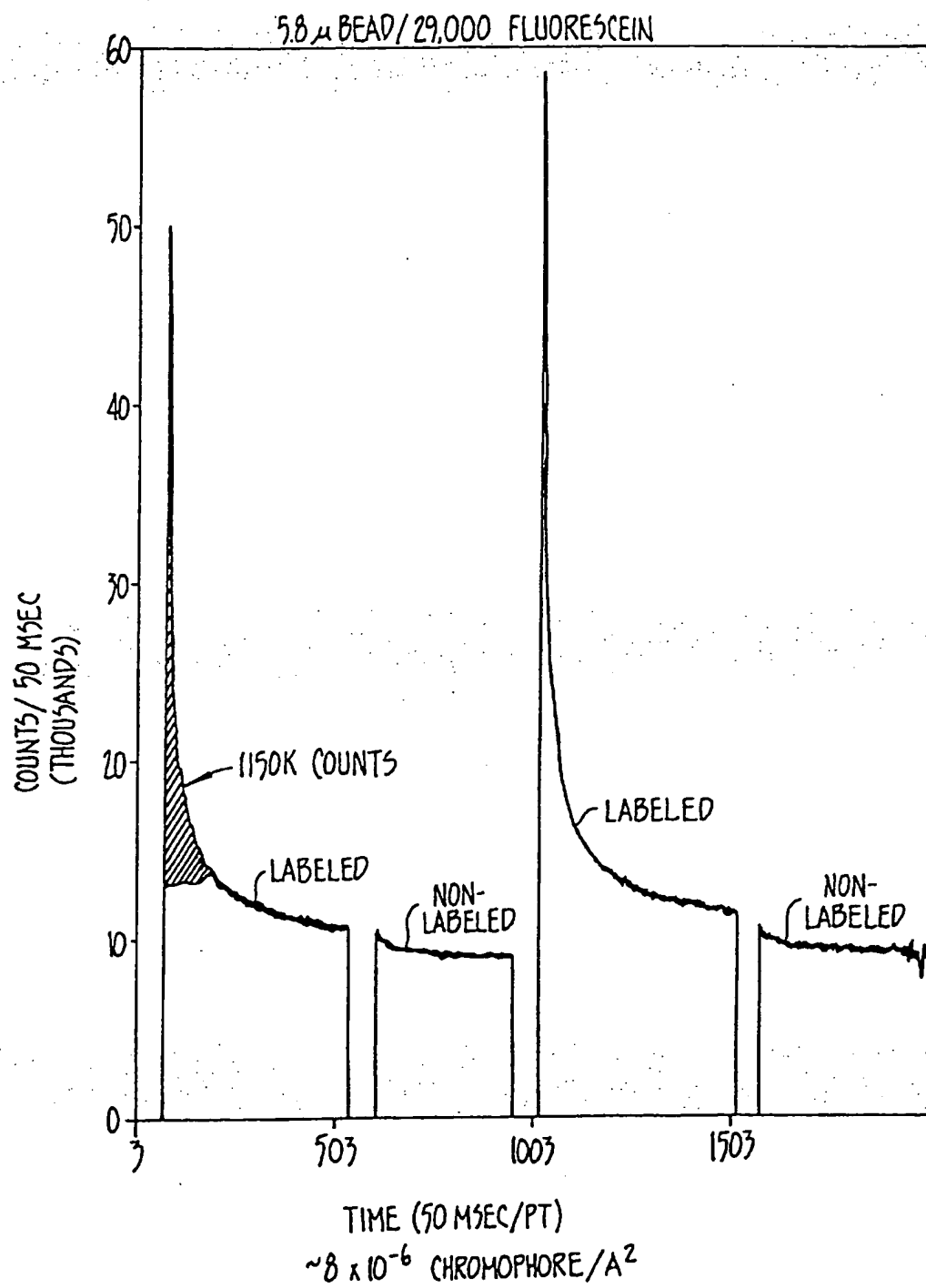


FIG. IIB.

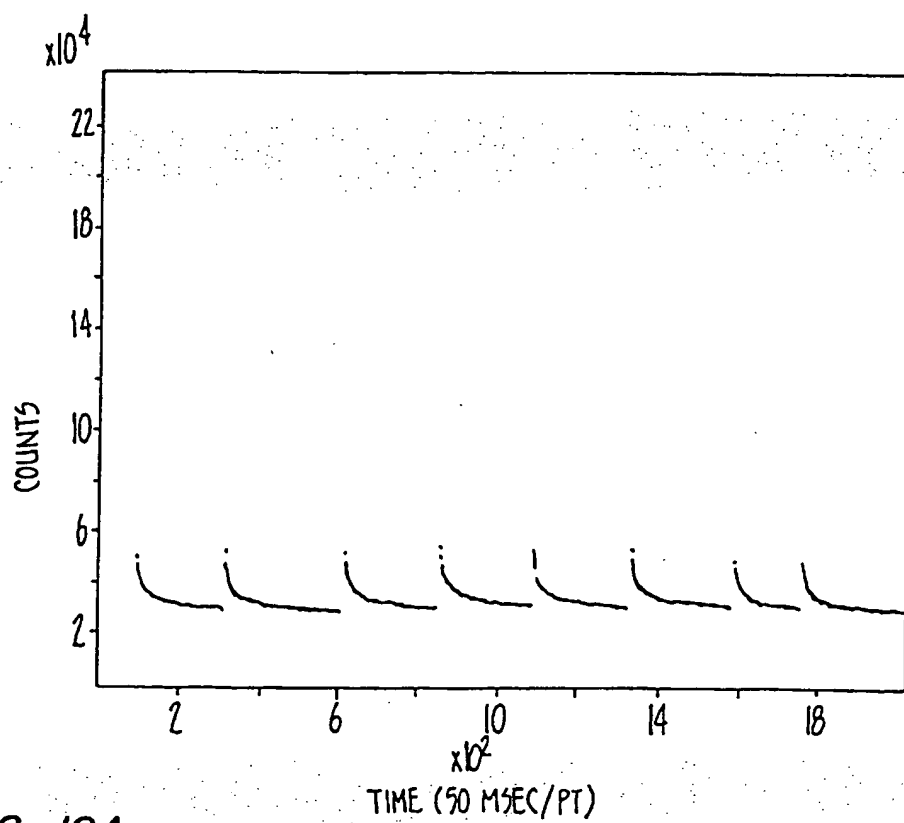


FIG. 12A.

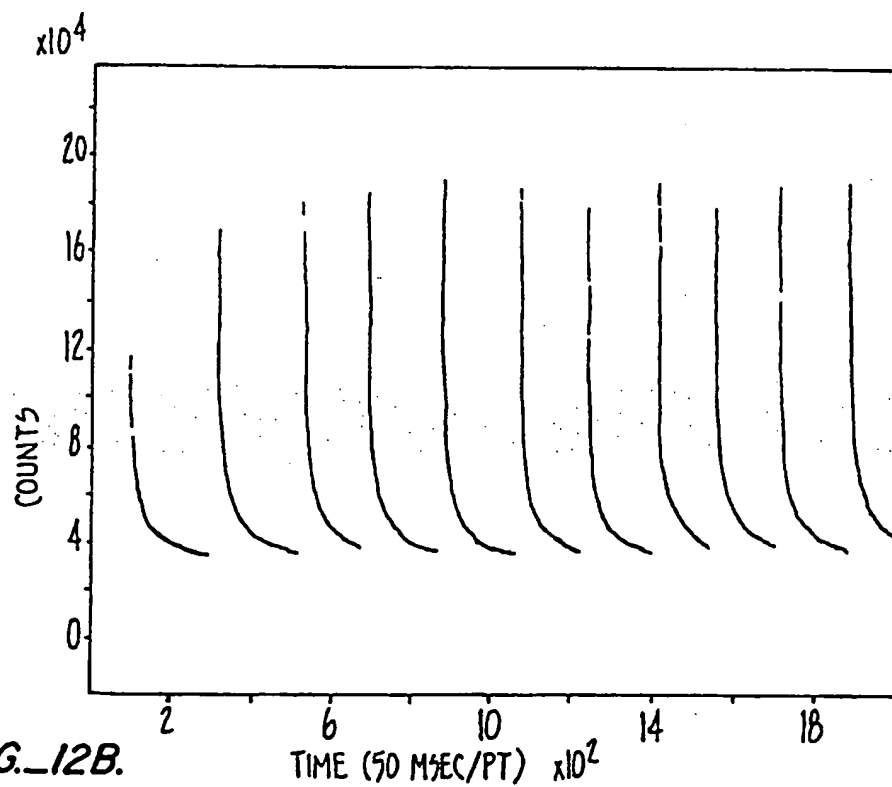
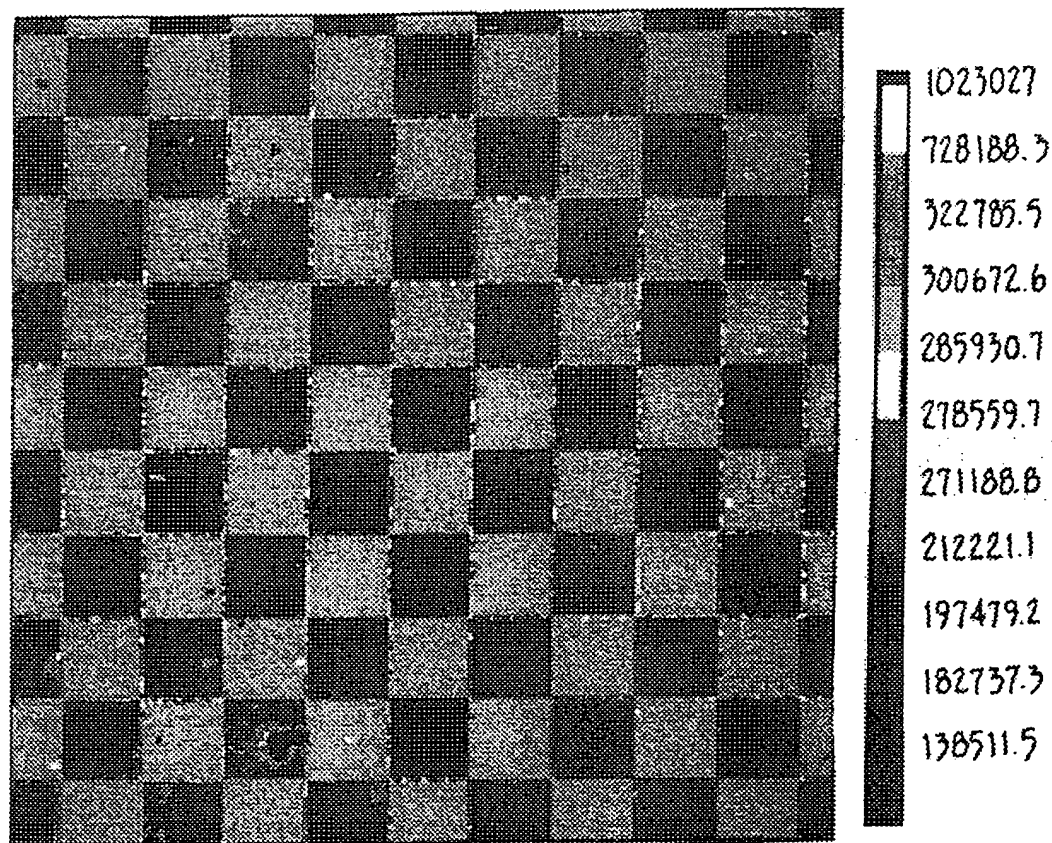
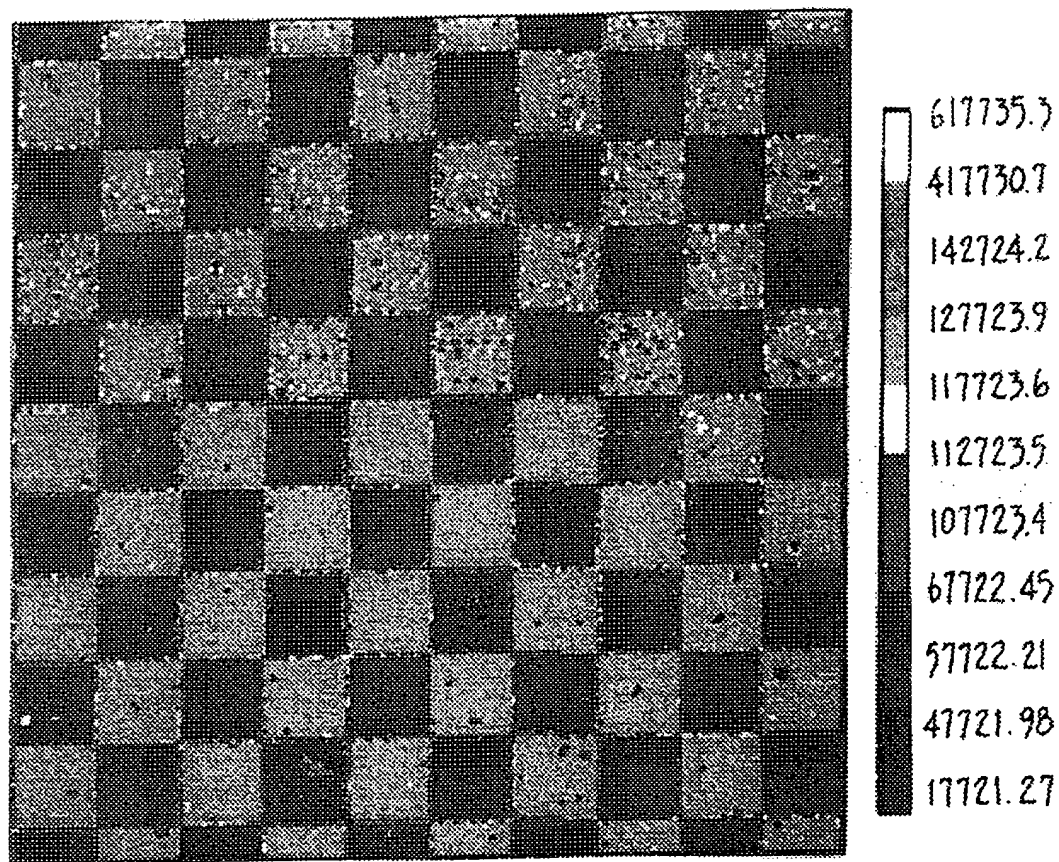


FIG. 12B.



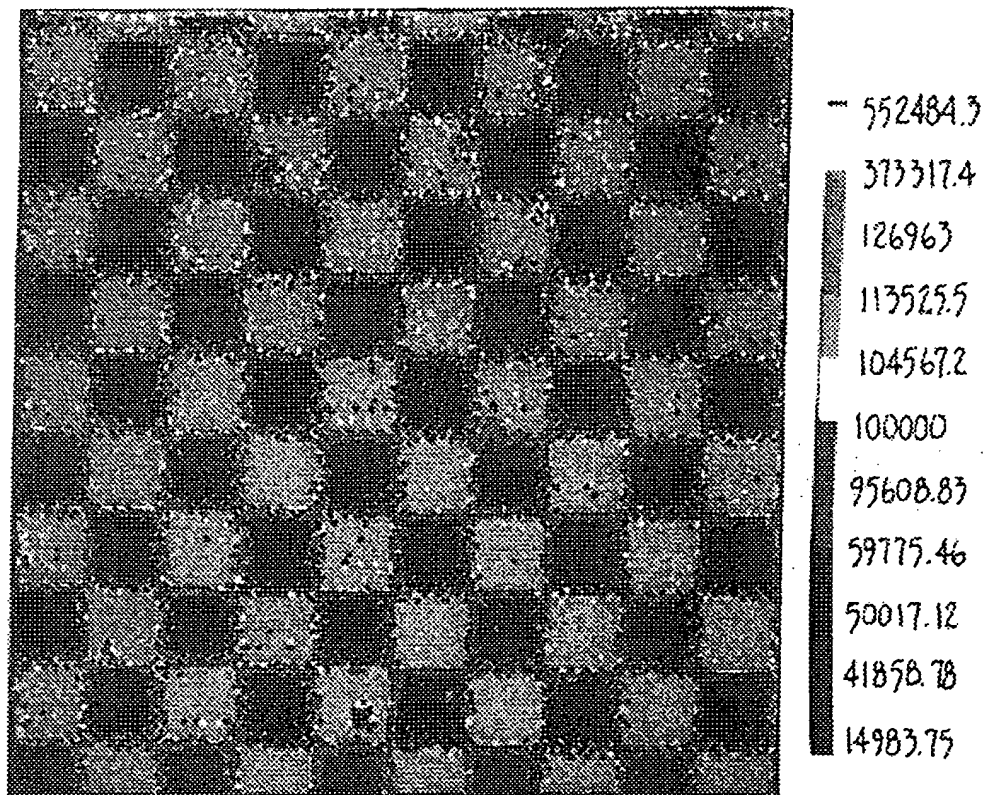
MEAN: 285930.7
VAR: 2.173242E+10
 σ : 147419.2

FIG. 13A.



MEAN: 117723.6
VAR: 1.000047E+10
 σ : 100002.3

FIG. 13B.



MEAN: 104567.2
VAR: 8.025189E+09
 σ : 89583.42

FIG. 13C.

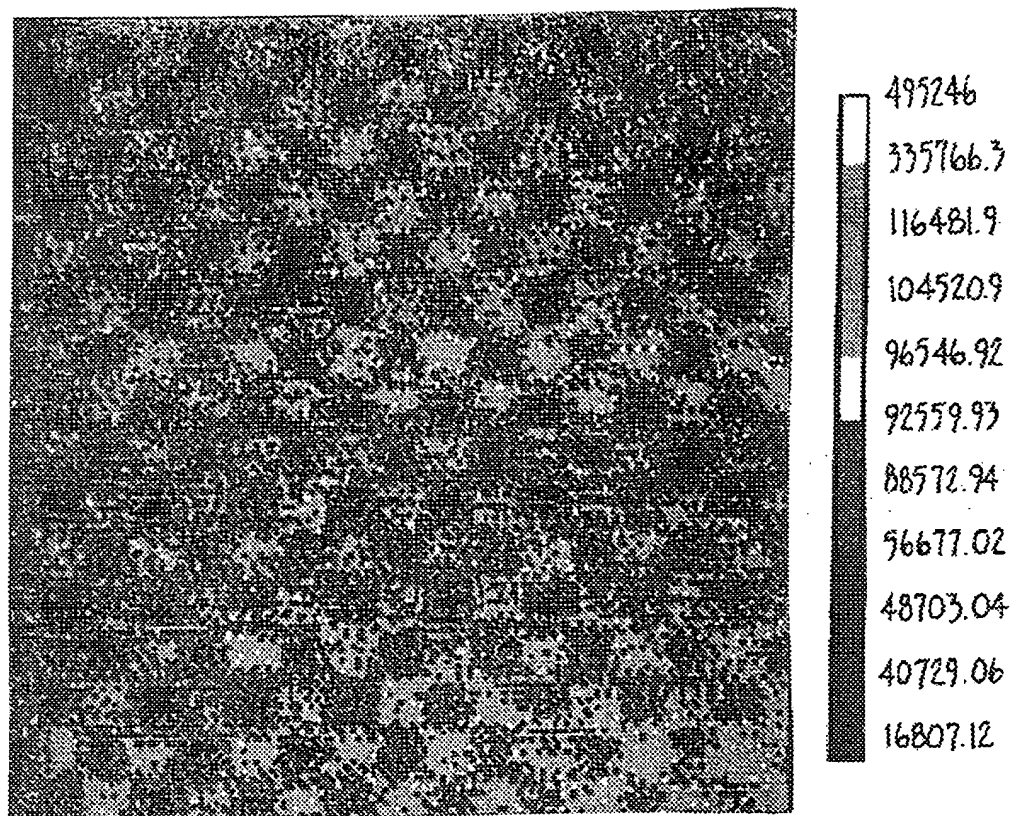


FIG. 13D.

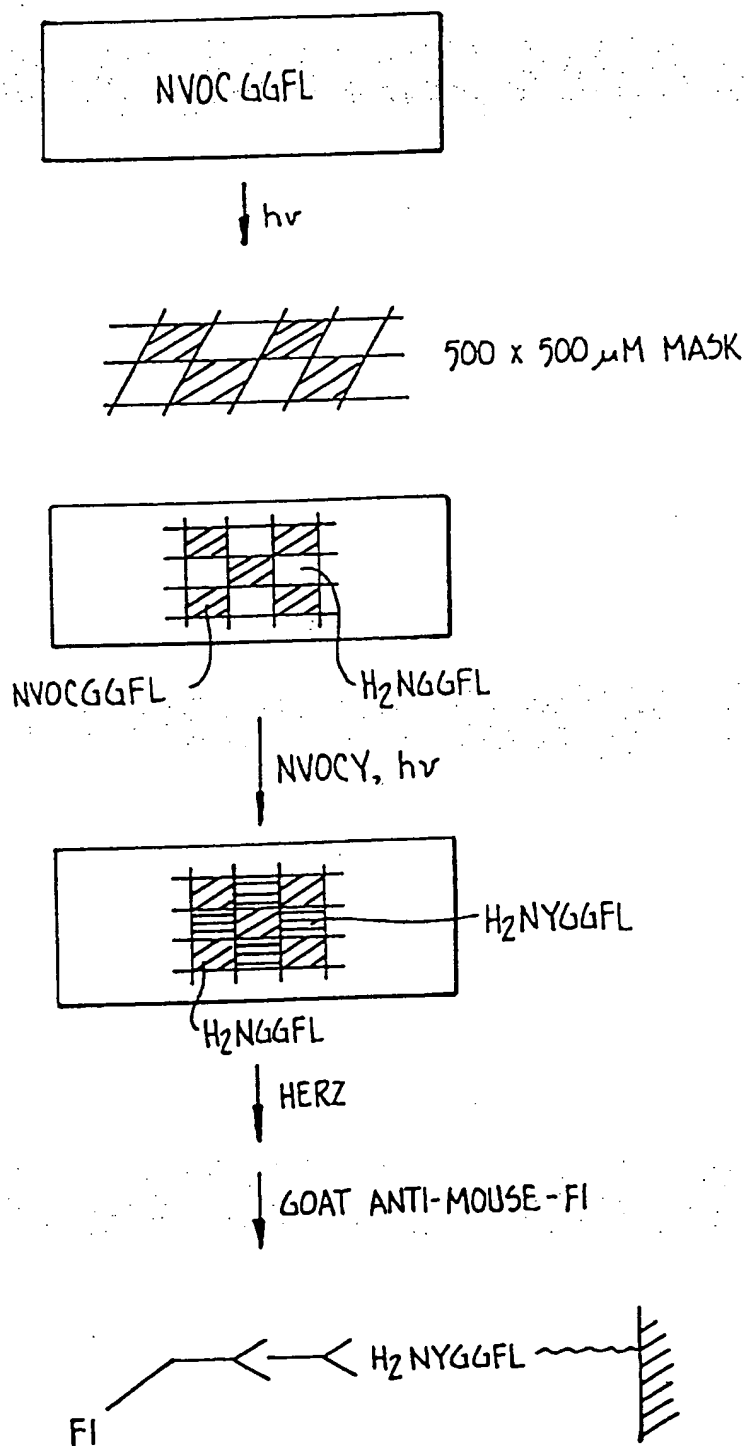


FIG. 14B.

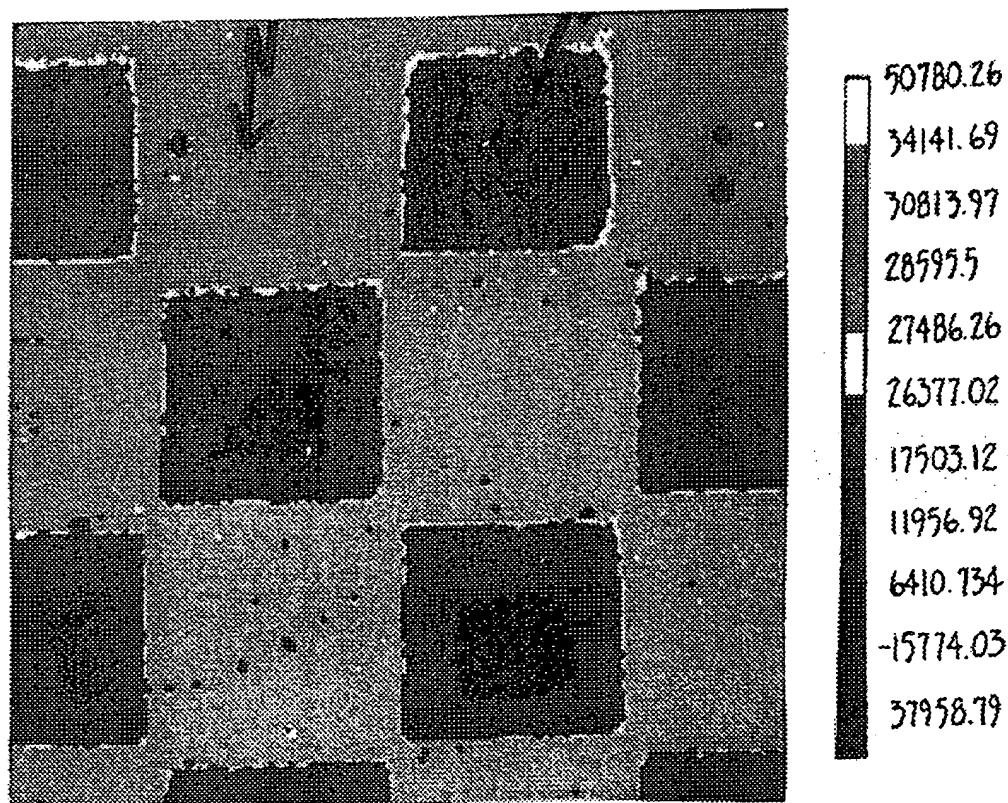


FIG. 15A.

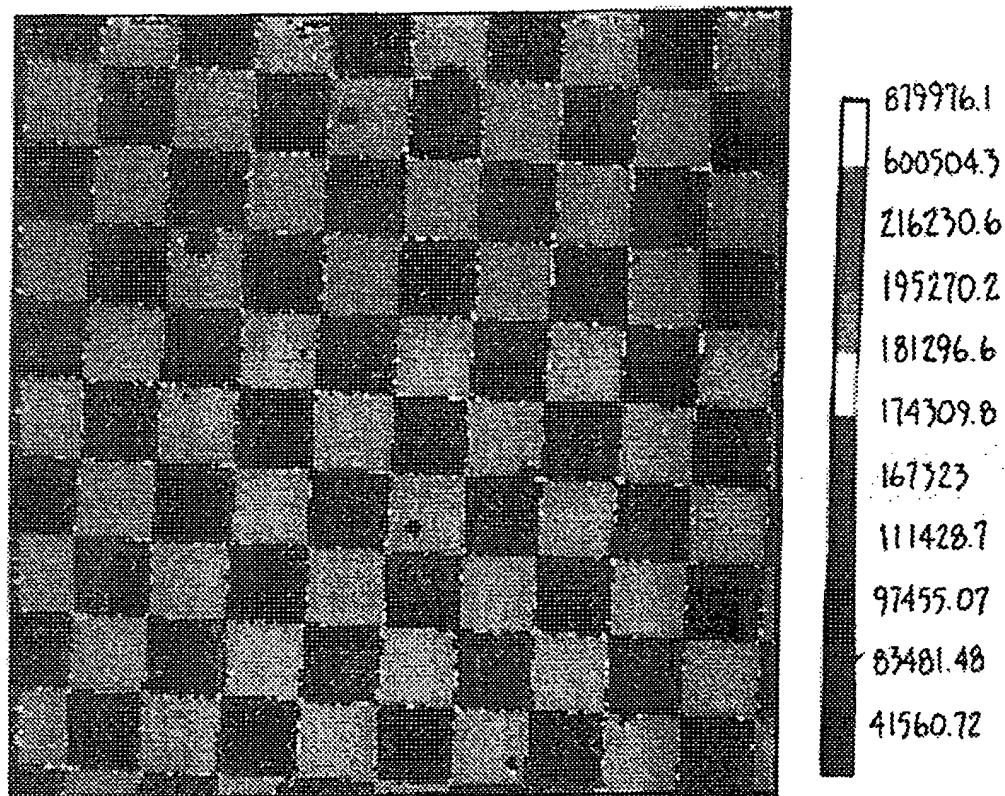
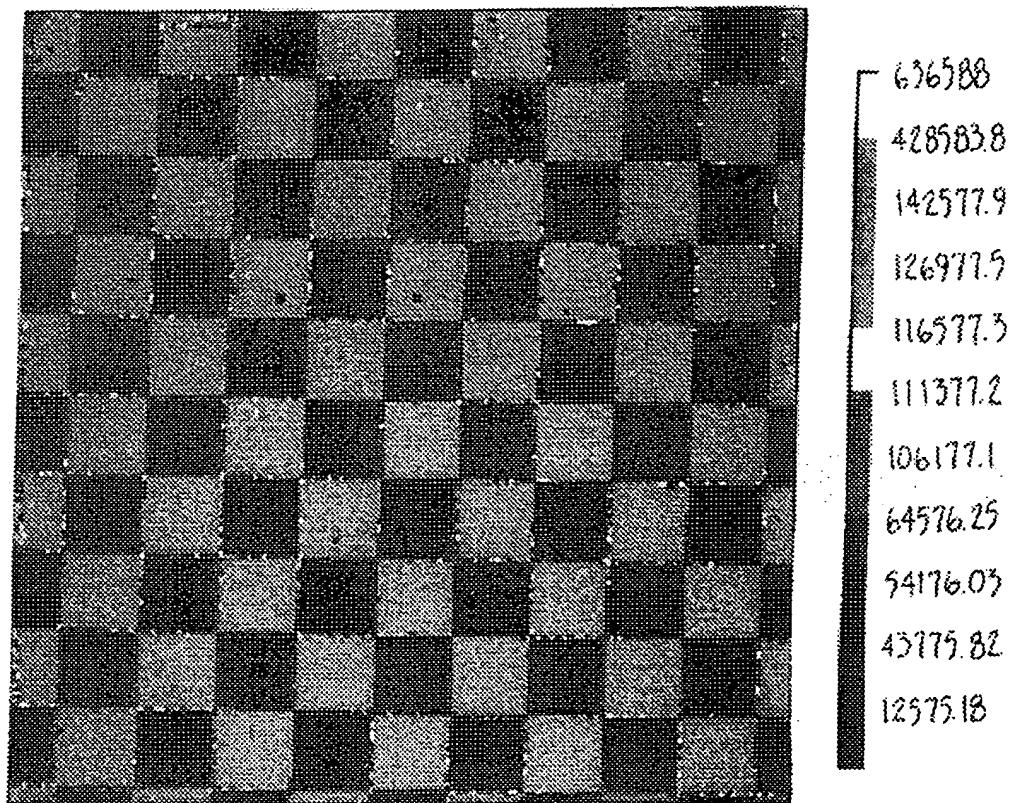
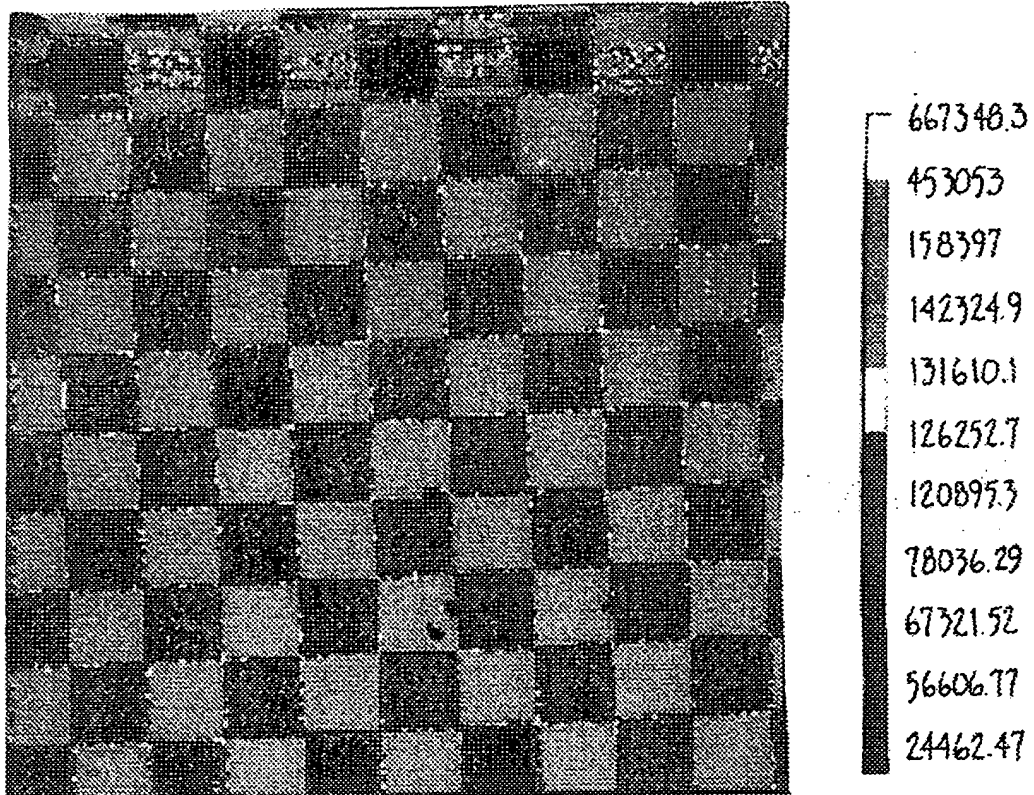


FIG. 15B.



MEAN: 116577.3
VAR: 1.081645E+10
σ: 104002.1

FIG. 16.



MEAN: 131610.1
VAR: 1.148062E+10
 σ : 107147.6

FIG. 17.

P	A	S	G	
<u>L</u> P _G FL	<u>L</u> A _G FL	<u>L</u> S _G FL	<u>L</u> G _G FL	L
<u>F</u> P _G FL	<u>F</u> A _G FL	<u>F</u> S _G FL	<u>F</u> G _G FL	F
<u>W</u> P _G FL	<u>W</u> A _G FL	<u>W</u> S _G FL	<u>W</u> G _G FL	W
<u>Y</u> P _G FL	<u>Y</u> A _G FL	<u>Y</u> S _G FL	<u>Y</u> G _G FL	Y

L SET

FIG. 18A.

p	a	s	G	
<u>Y</u> p _G FL	<u>Y</u> a _G FL	<u>Y</u> s _G FL	<u>Y</u> G _G FL	Y
<u>f</u> p _G FL	<u>f</u> a _G FL	<u>f</u> s _G FL	<u>f</u> G _G FL	f
<u>w</u> p _G FL	<u>w</u> a _G FL	<u>w</u> s _G FL	<u>w</u> G _G FL	w
<u>y</u> p _G FL	<u>y</u> a _G FL	<u>y</u> s _G FL	<u>y</u> G _G FL	y

D SET

FIG. 18B.

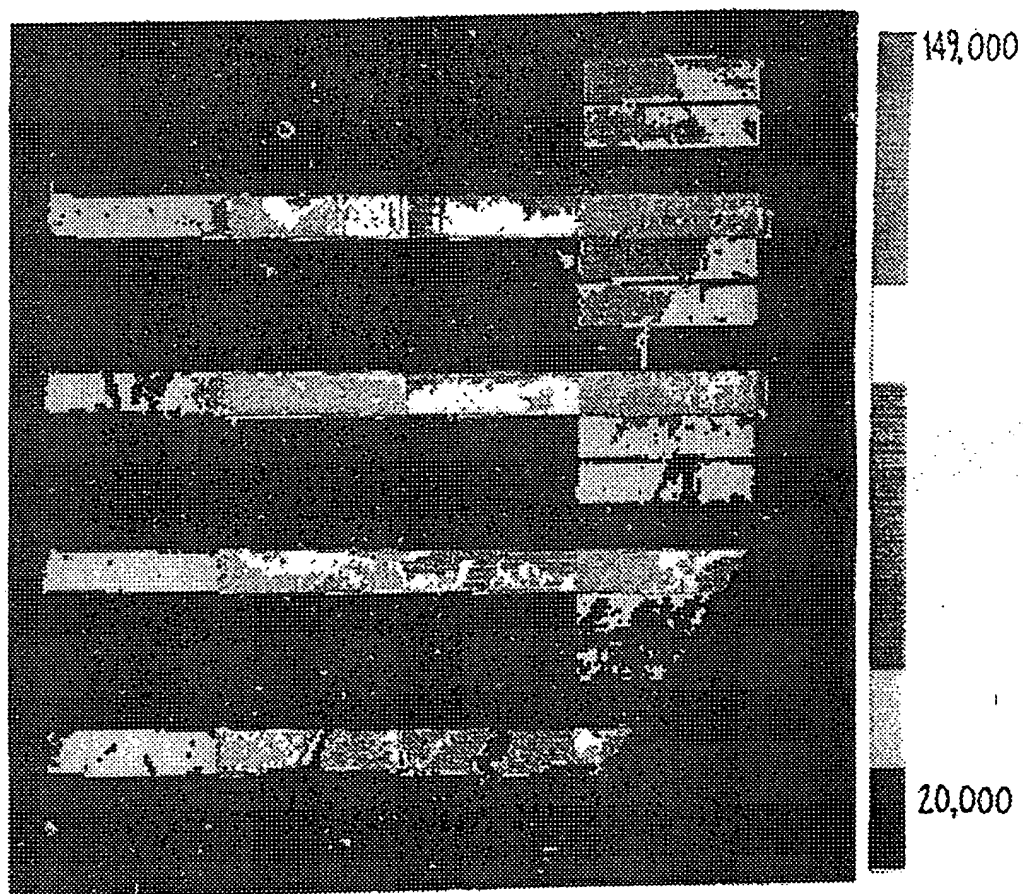


FIG. 19.

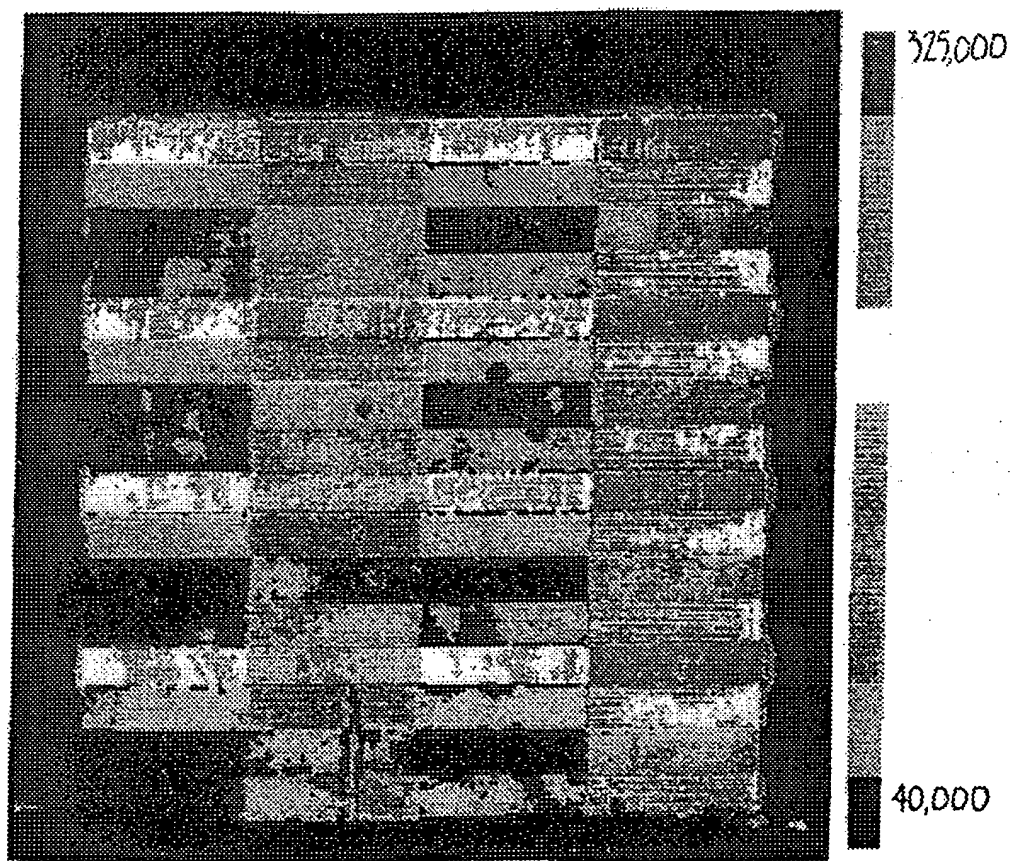


FIG. 20.

NUCLEIC ACID ARRAYS

This application is a continuation of Ser. No. 09/129,470 filed Aug. 4, 1998, which is a continuation of Ser. No. 08/456,598 filed Jun. 1, 1995, which is a divisional of Ser. No. 07/954,646 filed Sep. 30, 1992, now issued as U.S. Pat. No. 5,445,934, which is a divisional of Ser. No. 07/850,356, filed Mar. 12, 1992, now issued as U.S. Pat. No. 5,405,783, which is a divisional of Ser. No. 07/492,462 filed Mar. 7, 1990, now issued as U.S. Pat. No. 5,143,854, which is a continuation-in-part of Ser. No. 07/362,901 filed Jun. 7, 1989, now abandoned, the disclosures of which are incorporated by reference.

COPYRIGHT NOTICE

A portion of the disclosure of this patent document contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the Patent and Trademark Office patent file or records, but otherwise reserves all copyright rights whatsoever.

BACKGROUND OF THE INVENTION

The present inventions relate to the synthesis and placement materials at known locations. In particular, one embodiment of the inventions provides a method and associated apparatus for preparing diverse chemical sequences at known locations on a single substrate surface. The inventions may be applied, for example, in the field of preparation of oligomer, peptide, nucleic acid, oligosaccharide, phospholipid, polymer, or drug congener preparation, especially to create sources of chemical diversity for use in screening for biological activity.

The relationship between structure and activity of molecules is a fundamental issue in the study of biological systems. Structure-activity relationships are important in understanding, for example, the function of enzymes, the ways in which cells communicate with each other, as well as cellular control and feedback systems.

Certain macromolecules are known to interact and bind to other molecules having a very specific three-dimensional spatial and electronic distribution. Any large molecule having such specificity can be considered a receptor, whether it is an enzyme catalyzing hydrolysis of a metabolic intermediate, a cell-surface protein mediating membrane transport of ions, a glycoprotein serving to identify a particular cell to its neighbors, an IgG-class antibody circulating in the plasma, an oligonucleotide sequence of DNA in the nucleus, or the like. The various molecules which receptors selectively bind are known as ligands.

Many assays are available for measuring the binding affinity of known receptors and ligands, but the information which can be gained from such experiments is often limited by the number and type of ligands which are available. Novel ligands are sometimes discovered by chance or by application of new techniques for the elucidation of molecular structure, including x-ray crystallographic analysis and recombinant genetic techniques for proteins.

Small peptides are an exemplary system for exploring the relationship between structure and function in biology. A peptide is a sequence of amino acids. When the twenty naturally occurring amino acids are condensed into polymeric molecules they form a wide variety of three-dimensional configurations, each resulting from a particular amino acid sequence and solvent condition. The number of

possible pentapeptides of the 20 naturally occurring amino acids, for example, is 20^5 or 3.2 million different peptides. The likelihood that molecules of this size might be useful in receptor-binding studies is supported by epitope analysis studies showing that some antibodies recognize sequences as short as a few amino acids with high specificity. Furthermore, the average molecular weight of amino acids puts small peptides in the size range of many currently useful pharmaceutical products.

Pharmaceutical drug discovery is one type of research which relies on such a study of structure-activity relationships. In most cases, contemporary pharmaceutical research can be described as the process of discovering novel ligands with desirable patterns of specificity for biologically important receptors. Another example is research to discover new compounds for use in agriculture, such as pesticides and herbicides.

Sometimes, the solution to a rational process of designing ligands is difficult or unyielding. Prior methods of preparing large numbers of different polymers have been painstakingly slow when used at a scale sufficient to permit effective rational or random screening. For example, the "Merrifield" method (*J. Am. Chem. Soc.* (1963) 85:2149-2154, which is incorporated herein by reference for all purposes) has been used to synthesize peptides on a solid support. In the Merrifield method, an amino acid is covalently bonded to a support made of an insoluble polymer. Another amino acid with an alpha protected group is reacted with the covalently bonded amino acid to form a dipeptide. After washing, the protective group is removed and a third amino acid with an alpha protective group is added to the dipeptide. This process is continued until a peptide of a desired length and sequence is obtained. Using the Merrifield method, it is not economically practical to synthesize more than a handful of peptide sequences in a day.

To synthesize larger numbers of polymer sequences, it has also been proposed to use a series of reaction vessels for polymer synthesis. For example, a tubular reactor system may be used to synthesize a linear polymer on a solid phase support by automated sequential addition of reagents. This method still does not enable the synthesis of a sufficiently large number of polymer sequences for effective economical screening.

Methods of preparing a plurality of polymer sequences are also known in which a foraminous container encloses a known quantity of reactive particles, the particles being larger in size than foramina of the container. The containers may be selectively reacted with desired materials to synthesize desired sequences of product molecules. As with other methods known in the art, this method cannot practically be used to synthesize a sufficient variety of polypeptides for effective screening.

Other techniques have also been described. These methods include the synthesis of peptides on 96 plastic pins which fit the format of standard microtiter plates. Unfortunately, while these techniques have been somewhat useful, substantial problems remain. For example, these methods continue to be limited in the diversity of sequences which can be economically synthesized and screened.

From the above, it is seen that an improved method and apparatus for synthesizing a variety of chemical sequences at known locations is desired.

SUMMARY OF THE INVENTION

An improved method and apparatus for the preparation of a variety of polymers is disclosed.

In one preferred embodiment, linker molecules are provided on a substrate. A terminal end of the linker molecules is provided with a reactive functional group protected with a photoremovable protective group. Using lithographic methods, the photoremovable protective group is exposed to light and removed from the linker molecules in first selected regions. The substrate is then washed or otherwise contacted with a first monomer that reacts with exposed functional groups on the linker molecules. In a preferred embodiment, the monomer is an amino acid containing a photoremovable protective group at its amino or carboxy terminus and the linker molecule terminates in an amino or carboxy acid group bearing a photoremovable protective group.

A second set of selected regions is, thereafter, exposed to light and the photoremovable protective group on the linker molecule/protected amino acid is removed at the second set of regions. The substrate is then contacted with a second monomer containing a photoremovable protective group for reaction with exposed functional groups. This process is repeated to selectively apply monomers until polymers of a desired length and desired chemical sequence are obtained. Photolabile groups are then optionally removed and the sequence is, thereafter, optionally capped. Side chain protective groups, if present, are also removed.

By using the lithographic techniques disclosed herein, it is possible to direct light to relatively small and precisely known locations on the substrate. It is, therefore, possible to synthesize polymers of a known chemical sequence at known locations on the substrate.

The resulting substrate will have a variety of uses including, for example, screening large numbers of polymers for biological activity. To screen for biological activity, the substrate is exposed to one or more receptors such as antibody whole cells, receptors on vesicles, lipids, or any one of a variety of other receptors. The receptors are preferably labeled with, for example, a fluorescent marker, radioactive marker, or a labeled antibody reactive with the receptor. The location of the marker on the substrate is detected with, for example, photon detection or autoradiographic techniques. Through knowledge of the sequence of the material at the location where binding is detected, it is possible to quickly determine which sequence binds with the receptor and, therefore, the technique can be used to screen large numbers of peptides. Other possible applications of the inventions herein include diagnostics in which various antibodies for particular receptors would be placed on a substrate and, for example, blood sera would be screened for immune deficiencies. Still further applications include, for example, selective "doping" of organic materials in semiconductor devices, and the like.

In connection with one aspect of the invention an improved reactor system for synthesizing polymers is also disclosed. The reactor system includes a substrate mount which engages a substrate around a periphery thereof. The substrate mount provides for a reactor space between the substrate and the mount through or into which reaction fluids are pumped or flowed. A mask is placed on or focused on the substrate and illuminated so as to deprotect selected regions of the substrate in the reactor space. A monomer is pumped through the reactor space or otherwise contacted with the substrate and reacts with the deprotected regions. By selectively deprotecting regions on the substrate and flowing predetermined monomers through the reactor space, desired polymers at known locations may be synthesized.

Improved detection apparatus and methods are also disclosed. The detection method and apparatus utilize a sub-

strate having a large variety of polymer sequences at known locations on a surface thereof. The substrate is exposed to a fluorescently labeled receptor which binds to one or more of the polymer sequences. The substrate is placed in a microscope detection apparatus for identification of locations where binding takes place. The microscope detection apparatus includes a monochromatic or polychromatic light source for directing light at the substrate, means for detecting fluoresced light from the substrate, and means for determining a location of the fluoresced light. The means for detecting light fluoresced on the substrate may in some embodiments include a photon counter. The means for determining a location of the fluoresced light may include an x/y translation table for the substrate. Translation of the slide and data collection are recorded and managed by an appropriately programmed digital computer.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates masking and irradiation of a substrate at a first location. The substrate is shown in cross-section;

FIG. 2 illustrates the substrate after application of a monomer "A";

FIG. 3 illustrates irradiation of the substrate at a second location;

FIG. 4 illustrates the substrate after application of monomer "B";

FIG. 5 illustrates irradiation of the "A" monomer;

FIG. 6 illustrates the substrate after a second application of "B";

FIG. 7 illustrates a completed substrate;

FIGS. 8A and 8B illustrate alternative embodiments of a reactor system for forming a plurality of polymers on a substrate;

FIG. 9 illustrates a detection apparatus for locating fluorescent markers on the substrate;

FIGS. 10A-10M illustrate the method as it is applied to the production of the trimers of monomers "A" and "B";

FIGS. 11A and 11B are fluorescence traces for standard fluorescent beads;

FIGS. 12A and 12B are fluorescence curves for NVOC slides not exposed and exposed to light respectively;

FIGS. 13A to 13D are fluorescence plots of slides exposed through 100 μm , 50 μm , 20 μm , and 10 μm masks;

FIG. 14A and 14B illustrates fluorescence of a slide with the peptide YGGFL on selected regions of its surface which has been exposed to labeled Herz antibody specific for this sequence;

FIGS. 15A and 15D illustrate formation of and a fluorescence plot of a slide with a checkerboard pattern of YGGFL and GGFL exposed to labeled Herz antibody. FIG. 15A illustrates a 500x500 μm mask which has been focused on the substrate according to FIG. 8A while FIG. 15B illustrates a 50x50 μm mask placed in direct contact with the substrate in accord with FIG. 8B;

FIG. 16 is a fluorescence plot of YGGFL and PGGFL synthesized in a 50 μm checkerboard pattern;

FIG. 17 is a fluorescence plot of YPGFL and is YGGFL synthesized in a 50 μm checkerboard pattern;

FIGS. 18A and 18B illustrate the mapping of sixteen sequences synthesized on two different glass slides;

FIG. 19 is a fluorescence plot of the slide illustrated in FIG. 18A; and

FIG. 20 is a fluorescence plot of the slide illustrated in FIG. 10B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS

I. Glossary	
II. General	
III. Polymer Synthesis	
IV. Details of One Embodiment of a Reactor System	
V. Details of One Embodiment of a Fluorescent Detection Device	15
VI. Determination of Relative Binding Strength of Receptors	
VII. Examples	20
A. Slide Preparation	
B. Synthesis of Eight Trimers of "A" and "B"	
C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group	
D. Demonstration of Signal Capability	25
E. Determination of the Number of Molecules Per Unit Area	
F. Removal of NVOC and Attachment of a Fluorescent Marker	
G. Use of a Mask in Removal of NVOC	30
H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse	
I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody	
J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL	35
K. Monomer-by Monomer Synthesis of YGGFL and YPGGFL	
L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody	40
VIII. Illustrative Alternative Embodiment	
IX. Conclusion	

I. Glossary

The following terms are intended to have the following general meanings as they are used herein:

1. **Complementary:** Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.
2. **Epitope:** The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.
3. **Ligand:** A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.
4. **Monomer:** A member of the set of small molecules which can be joined together to form a polymer. The set of

monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer.

5. **Peptide:** A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer. Peptides are more than two amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, *Biochemistry*, Third Ed., 1988, which is incorporated herein by reference for all purposes.
6. **Radiation:** Energy which may be selectively applied including energy having a wavelength of between 10^{-14} and 10^4 meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultra-violet radiation, visible light, infrared radiation, microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.
7. **Receptor:** A molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

Other examples of receptors which can be investigated by this invention include but are not restricted to:

- a) **Microorganism receptors:** Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful in a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.
- b) **Enzymes:** For instance, the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters; determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.
- c) **Antibodies:** For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of

- vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases (e.g., by blocking the binding of the "self" antibodies).
- d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.
 - e) Catalytic Polypeptides: Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. application Ser. No. 404,920, which is incorporated herein by reference for all purposes.
 - f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which bind to a receptor may lead to the development of drugs to control blood pressure.
 - g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.
8. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.
9. Protective Group: A material which is bound to a monomer unit and which may be spatially removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protective groups with utility herein include Nitroveratryloxy carbonyl, Nitrobenzyloxy carbonyl, Dimethyl dimethoxybenzyloxy carbonyl, 5-Bromo-7-nitroindoliny, o-Hydroxy- α -methyl cinnamoyl, and 2-oxymethylene anthraquinone. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.
10. Predefined Region: A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."
11. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result

of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.

II. General

The present invention provides methods and apparatus for the preparation and use of a substrate having a plurality of polymer sequences in predefined regions. The invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α -, β -, or ω -amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides.

The prepared substrate may, for example, be used in screening a variety of polymers as ligands for binding with a receptor, although it will be apparent that the invention could be used for the synthesis of a receptor for binding with a ligand. The substrate disclosed herein will have a wide variety of other uses. Merely by way of example, the invention herein can be used in determining peptide and nucleic acid sequences which bind to proteins, finding sequence-specific binding drugs, identifying epitopes recognized by antibodies, and evaluation of a variety of drugs for clinical and diagnostic applications, as well as combinations of the above.

The invention preferably provides for the use of a substrate "S" with a surface. Linker molecules "L" are optionally provided on a surface of the substrate. The purpose of the linker molecules, in some embodiments, is to facilitate receptor recognition of the synthesized polymers.

Optionally, the linker molecules may be chemically protected for storage purposes. A chemical storage protective group such as t-BOC (t-butoxycarbonyl) may be used in some embodiments. Such chemical protective groups would be chemically removed upon exposure to, for example, acidic solution and would serve to protect the surface during storage and be removed prior to polymer preparation.

On the substrate or a distal end of the linker molecules, a functional group with a protective group P_0 is provided. The protective group P_0 may be removed upon exposure to radiation, electric fields, electric currents, or other activators to expose the functional group.

In a preferred embodiment, the radiation is ultraviolet (UV), infrared (IR), or visible light. As more fully described below, the protective group may alternatively be an electrochemically-sensitive group which may be removed in the presence of an electric field. In still further alternative embodiments, ion beams, electron beams, or the like may be used for deprotection.

In some embodiments, the exposed regions and, therefore, the area upon which each distinct polymer sequence is synthesized are smaller than about 1 cm^2 or less than 1 mm^2 . In preferred embodiments the exposed area is less than about $10,000 \mu\text{m}^2$ or, more preferably, less than $100 \mu\text{m}^2$ and may, in some embodiments, encompass the binding site for as few as a single molecule. Within these regions, each polymer is preferably synthesized in a substantially pure form.

Concurrently or after exposure of a known region of the substrate to light, the surface is contacted with a first

monomer unit M_1 which reacts with the functional group which has been exposed by the deprotection step. The first monomer includes a protective group P_1 . P_1 may or may not be the same as P_0 .

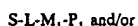
Accordingly, after a first cycle, known first regions of the surface may comprise the sequence:



while remaining regions of the surface comprise the sequence:

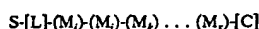


Thereafter, second regions of the surface (which may include the first region) are exposed to light and contacted with a second monomer M_2 (which may or may not be the same as M_1) having a protective group P_2 . P_2 may or may not be the same as P_0 and P_1 . After this second cycle, different regions of the substrate may comprise one or more of the following sequences:



The above process is repeated until the substrate includes desired polymers of desired lengths. By controlling the locations of the substrate exposed to light and the reagents exposed to the substrate following exposure, the location of each sequence will be known.

Thereafter, the protective groups are removed from some or all of the substrate and the sequences are, optionally, capped with a capping unit C. The process results in a substrate having a surface with a plurality of polymers of the following general formula:



where square brackets indicate optional groups, and $M_1 \dots M_x$ indicates any sequence of monomers. The number of monomers could cover a wide variety of values, but in a preferred embodiment they will range from 2 to 100.

In some embodiments a plurality of locations on the substrate polymers are to contain a common monomer subsequence. For example, it may be desired to synthesize a sequence $S-M_1-M_2-M_3$ at first locations and a sequence $S-M_4-M_2-M_3$ at second locations. The process would commence with irradiation of the first locations followed by contacting with M_1-P , resulting in the sequence $S-M_1-P$ at the first location. The second locations would then be irradiated and contacted with M_4-P , resulting in the sequence $S-M_4-P$ at the second locations. Thereafter both the first and second locations would be irradiated and contacted with the dimer M_2-M_3 , resulting in the sequence $S-M_1-M_2-M_3$ at the first locations and $S-M_4-M_2-M_3$ at the second locations. Of course, common subsequences of any length could be utilized including those in a range of 2 or more monomers, 2 to 100 monomers, 2 to 20 monomers, and a most preferred range of 2 to 3 monomers.

According to other embodiments, a set of masks is used for the first monomer layer and, thereafter, varied light wavelengths are used for selective deprotection. For example, in the process discussed above, first regions are first exposed through a mask and reacted with a first monomer having a first protective group P_1 , which is removable

upon exposure to a first wavelength of light (e.g., IR). Second regions are masked and reacted with a second monomer having a second protective group P_2 , which is removable upon exposure to a second wavelength of light (e.g., UV). Thereafter, masks become unnecessary in the synthesis because the entire substrate may be exposed alternatively to the first and second wavelengths of light in the deprotection cycle.

The polymers prepared on a substrate according to the above methods will have a variety of uses including, for example, screening for biological activity. In such screening activities, the substrate containing the sequences is exposed to an unlabeled or labeled receptor such as an antibody, receptor on a cell, phospholipid vesicle, or any one of a variety of other receptors. In one preferred embodiment the polymers are exposed to a first, unlabeled receptor of interest and, thereafter, exposed to a labeled receptor-specific recognition element, which is, for example, an antibody. This process will provide signal amplification in the detection stage.

The receptor molecules may bind with one or more polymers on the substrate. The presence of the labeled receptor and, therefore, the presence of a sequence which binds with the receptor is detected in a preferred embodiment through the use of autoradiography, detection of fluorescence with a charge-coupled device, fluorescence microscopy, or the like. The sequence of the polymer at the locations where the receptor binding is detected may be used to determine all or part of a sequence which is complementary to the receptor.

Use of the invention herein is illustrated primarily with reference to screening for biological activity. The invention will, however, find many other uses. For example, the invention may be used in information storage (e.g., on optical disks), production of molecular electronic devices, production of stationary phases in separation sciences, production of dyes and brightening agents, photography, and in immobilization of cells, proteins, lectins, nucleic acids, polysaccharides and the like in patterns on a surface via molecular recognition of specific polymer sequences. By synthesizing the same compound in adjacent, progressively differing concentrations, a gradient will be established to control chemotaxis or to develop diagnostic dipsticks which, for example, titrate an antibody against an increasing amount of antigen. By synthesizing several catalyst molecules in close proximity, more efficient multistep conversions may be achieved by "coordinate immobilization." Coordinate immobilization also may be used for electron transfer systems, as well as to provide both structural integrity and other desirable properties to materials such as lubrication, wetting, etc.

According to alternative embodiments, molecular biodistribution or pharmacokinetic properties may be examined. For example, to assess resistance to intestinal or serum proteases, polymers may be capped with a fluorescent tag and exposed to biological fluids of interest.

III. Polymer Synthesis

FIG. 1 illustrates one embodiment of the invention disclosed herein in which a substrate 2 is shown in cross-section. Essentially, any conceivable substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The

substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO₂, SiN₄, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface relief features of less than 10 Å.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate in accord with the teaching of copending application Ser. No. 404,920, previously incorporated herein by reference. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si—OH functionalities, such as are found on silica surfaces.

The surface 4 of the substrate is preferably provided with a layer of linker molecules 6, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6–50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2–10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other linker molecules may be used in light of this disclosure.

According to alternative embodiments, the linker molecules are selected based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to more closely approach the synthesized polymer.

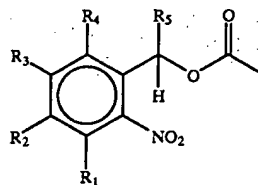
According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)trifluorochloroethylene surfaces, or preferably, by siloxane

bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzyloxycarbonyl. In a preferred embodiment, 6-nitroveratryloxy-carbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or α,α -dimethyldimethoxybenzyloxycarbonyl (DDZ) is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:



where R₁ is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R₂ is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R₃ is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R₄ is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R₅ is alkyl, alkenyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include o-hydroxy- α -methyl cinnamoyl derivatives. Photoremovable protective groups are described in, for example, Patchornik, *J. Am. Chem. Soc.* (1970) 92:6333 and Amit et al., *J. Org. Chem.* (1974) 39:192, both of which are incorporated herein by reference.

In an alternative embodiment the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinnamate group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in light of this disclosure.

As shown in FIG. 1, the linking molecules are preferably exposed to, for example, light through a suitable mask 8 using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze,

VLSI Technology, McGraw-Hill (1983), and Mead et al., *Introduction to VLSI Systems*, Addison-Wesley (1980), which are incorporated herein by reference for all purposes. The light may be directed at either the surface containing the protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups. In the embodiment shown in FIG. 1, light is directed at the surface of the substrate containing the protective groups. FIG. 1 illustrates the use of such masking techniques as they are applied to a positive reactive group so as to activate linking molecules and expose functional groups in areas 10a and 10b.

The mask 8 is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with, imaged on, or brought directly into contact with the substrate surface as shown in FIG. 1. "Openings" in the mask correspond to locations on the substrate where it is desired to remove photoremovable protective groups from the substrate. Alignment may be performed using conventional alignment techniques in which alignment marks (not shown) are used to accurately overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one described in Flanders et al., "A New Interferometric Alignment Technique," *App. Phys. Lett.* (1977) 31:426-428, which is incorporated herein by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazid or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps. Several reactive groups along with their corresponding wavelengths for deprotection are provided in Table 1.

TABLE 1

Group	Approximate Deprotection Wavelength
Nitroveratryloxy carbonyl (NVOC)	UV (300-400 nm)
Nitrobenzyloxy carbonyl (NBVC)	UV (300-350 nm)
Dimethyl dimethoxybenzyloxy carbonyl	UV (280-300 nm)
5-Bromo-7-nitroindolyl	UV (420 nm)
o-Hydroxy- α -methyl cinnamoyl	UV (300-350 nm)
2-Oxymethylene anthraquinone	UV (350 nm)

While the invention is illustrated primarily herein by way of the use of a mask to illuminate selected regions the substrate, other techniques may also be used. For example, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Pat. No. 4,719,615 (Feyrer et al.), which is incorporated herein by reference. In alternative embodiments a laser galvanometric scanner is utilized. In other

embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled so as to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The substrate may be irradiated either in contact or not in contact with a solution (not shown) and is, preferably, irradiated in contact with a solution. The solution contains reagents to prevent the by-products formed by irradiation from interfering with synthesis of the polymer according to some embodiments. Such by-products might include, for example, carbon dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions. Alternatively, the solution may contain reagents used to match the index of refraction of the substrate. Reagents added to the solution may further include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH) or reagents known to react with a given functional group (e.g., aryl nitroso+glyoxylic acid \rightarrow aryl formhydroxamate+CO₂).

Either concurrently with or after the irradiation step, the linker molecules are washed or otherwise contacted with a first monomer, illustrated by "A" in regions 12a and 12b in FIG. 2. The first monomer reacts with the activated functional groups of the linkage molecules which have been exposed to light. The first monomer, which is preferably an amino acid, is also provided with a photoprotective group. The photoprotective group on the monomer may be the same as or different than the protective group used in the linkage molecules, and may be selected from any of the above-described protective groups. In one embodiment, the protective groups for the A monomer is selected from the group NBOC and NVOC.

As shown in FIG. 3, the process of irradiating is thereafter repeated, with a mask repositioned so as to remove linkage protective groups and expose functional groups in regions 14a and 14b which are illustrated as being regions which were protected in the previous masking step. As an alternative to repositioning of the first mask, in many embodiments a second mask will be utilized. In other alternative embodiments, some steps may provide for illuminating a common region in successive steps. As shown in FIG. 3, it may be desirable to provide separation between irradiated regions. For example, separation of about 1-5 μ m may be appropriate to account for alignment tolerances.

As shown in FIG. 4, the substrate is then exposed to a second protected monomer "B," producing B regions 16a and 16b. Thereafter, the substrate is again masked so as to remove the protective groups and expose reactive groups on A region 12a and B region 16b. The substrate is again exposed to monomer B, resulting in the formation of the structure shown in FIG. 6. The dimers B-A and B-B have been produced on the substrate.

A subsequent series of masking and contacting steps similar to those described above with A (not shown) provides the structure shown in FIG. 7. The process provides all possible dimers of B and A, i.e., B-A, A-B, A-A, and B-B.

The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes, may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment the regions 12 and 16 on the substrate will have a surface area of between about 1 cm² and 10⁻¹⁰

15

cm². In some embodiments the regions 12 and 16 have areas of less than about 10⁻¹ cm², 10⁻² cm², 10⁻³ cm², 10⁻⁴ cm², 10⁻⁵ cm², 10⁻⁶ cm², 10⁻⁷ cm², 10⁻⁸ cm², or 10⁻¹⁰ cm². In a preferred embodiment, the regions 12 and 16 are between about 10×10 μm and 500×500 μm.

In some embodiments a single substrate supports more than about 10 different monomer sequences and preferably more than about 100 different monomer sequences, although in some embodiments more than about 10³, 10⁴, 10⁵, 10⁶, 10⁷, or 10⁸ different sequences are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% pure.

According to some embodiments, several sequences are intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated.

IV. Details of One Embodiment of a Reactor System

FIG. 8A schematically illustrates a preferred embodiment of a reactor system 100 for synthesizing polymers on the prepared substrate in accordance with one aspect of the invention. The reactor system includes a body 102 with a cavity 104 on a surface thereof. In preferred embodiments the cavity 104 is between about 50 and 1000 μm deep with a depth of about 500 μm preferred.

The bottom of the cavity is preferably provided with an array of ridges 106 which extend both into the plane of the Figure and parallel to the plane of the Figure. The ridges are preferably about 50 to 200 μm deep and spaced at about 2 to 3mm. The purpose of the ridges is to generate turbulent flow for better mixing. The bottom surface of the cavity is preferably light absorbing so as to prevent reflection of impinging light.

A substrate 112 is mounted above the cavity 104. The substrate is provided along its bottom surface 114 with a photoremovable protective group such as NVOC with or without an intervening linker molecule. The substrate is preferably transparent to a wide spectrum of light, but in some embodiments is transparent only at a wavelength at which the protective group may be removed (such as UV in the case of NVOC). The substrate in some embodiments is a conventional microscope glass slide or cover slip. The substrate is preferably as thin as possible, while still providing adequate physical support. Preferably, the substrate is less than about 1 mm thick, more preferably less than 0.5 mm thick, more preferably less than 0.1 mm thick, and most preferably less than 0.05 mm thick. In alternative preferred embodiments, the substrate is quartz or silicon.

The substrate and the body serve to seal the cavity except for an inlet port 108 and an outlet port 110. The body and the substrate may be mated for sealing in some embodiments with one or more gaskets. According to a preferred embodiment, the body is provided with two concentric gaskets and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets.

Fluid is pumped through the inlet port into the cavity by way of a pump 116 which may be, for example, a model no. B-120-S made by Eldex Laboratories. Selected fluids are circulated into the cavity by the pump, through the cavity, and out the outlet for recirculation or disposal. The reactor may be subjected to ultrasonic radiation and/or heated to aid in agitation in some embodiments.

16

Above the substrate 112, a lens 120 is provided which may be, for example, a 2" 100 mm focal length fused silica lens. For the sake of a compact system, a reflective mirror 122 may be provided for directing light from a light source 124 onto the substrate. Light source 124 may be, for example, a Xe(Hg) light source manufactured by Oriel and having model no. 66024. A second lens 126 may be provided for the purpose of projecting a mask image onto the substrate in combination with lens 112. This form of lithography is referred to herein as projection printing. As will be apparent from this disclosure, proximity printing and the like may also be used according to some embodiments.

Light from the light source is permitted to reach only selected locations on the substrate as a result of mask 128. Mask 128 may be, for example, a glass slide having etched chrome thereon. The mask 128 in one embodiment is provided with a grid of transparent locations and opaque locations. Such masks may be manufactured by, for example, Photo Sciences, Inc. Light passes freely through the transparent regions of the mask, but is reflected from or absorbed by other regions. Therefore, only selected regions of the substrate are exposed to light.

As discussed above, light valves (LCD's) may be used as an alternative to conventional masks to selectively expose regions of the substrate. Alternatively, fiber optic faceplates such as those available from Schott Glass, Inc. may be used for the purpose of contrast enhancement of the mask or as the sole means of restricting the region to which light is applied. Such faceplates would be placed directly above or on the substrate in the reactor shown in FIG. 8A. In still further embodiments, flys-eye lenses, tapered fiber optic faceplates, or the like, may be used for contrast enhancement.

In order to provide for illumination of regions smaller than a wavelength of light, more elaborate techniques may be utilized. For example, according to one preferred embodiment, light is directed at the substrate by way of molecular microcrystals on the tip of, for example, micropipettes. Such devices are disclosed in Lieberman et al., "A Light Source Smaller Than the Optical Wavelength," *Science* (1990) 247:59-61, which is incorporated herein by reference for all purposes.

In operation, the substrate is placed on the cavity and sealed thereto. All operations in the process of preparing the substrate are carried out in a room lit primarily or entirely by light of a wavelength outside of the light range at which the protective group is removed. For example, in the case of NVOC, the room should be lit with a conventional dark room light which provides little or no UV light. All operations are preferably conducted at about room temperature.

A first, deprotection fluid (without a monomer) is circulated through the cavity. The solution preferably is of 5 mM sulfuric acid in dioxane solution which serves to keep exposed amino groups protonated and decreases their reactivity with photolysis by-products. Absorptive materials such as N,N-diethylamino 2,4-dinitrobenzene, for example, may be included in the deprotection fluid which serves to absorb light and prevent reflection and unwanted photolysis.

The slide is, thereafter, positioned in a light raypath from the mask such that first locations on the substrate are illuminated and, therefore, deprotected. In preferred embodiments the substrate is illuminated for between about 1 and 15 minutes with a preferred illumination time of about 10 minutes at 10-20 mW/cm² with 365 nm light. The slides are neutralized (i.e., brought to a pH of about 7) after photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated

in bulk, and then reinstalled in the flow cell. Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump 116. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its α -nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container 118, through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2 provides an illustration of a mixture which may be used for solution A.

TABLE 2

Representative Monomer Carrier Solution "A"
100 mg NVOC amino protected amino acid
37 mg HOBT (1-Hydroxybenzotriazole)
250 μ l DMF (Dimethylformamide)
86 μ l DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500 μ l are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

TABLE 3

Representative Monomer Carrier Solution "B"
250 μ l DXF
111 mg BOP (Benzotriazolyl-n-oxy-tris(dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of removing the slide from the reactor and submersing it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50 \times times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light 124 is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example,

a solution of about 1% BSA (bovine serum albumin), 0.5% Tween in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4 μ g/ml.

FIG. 8B illustrates an alternative preferred embodiment of the reactor shown in FIG. 8A. According to this embodiment, the mask 128 is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the imaging lenses 120 and 126 are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

If, for example, it is desired to synthesize all 16 dinucleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is coupled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

TABLE 4

Mask Strategy Program

```

DEFINT A-Z
DIM b(20), w(20), l(500)
FS = "LPT1:"
OPEN FS FOR OUTPUT AS #1
jmax = 3      'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5      'Number of building blocks for res 1, 2, 3
g = 1: lmax(1) = 1
FOR j = 1 TO jmax: g = g * b(j): NEXT j
w(0) = 0: w(1) = g/b(1)
PRINT #1, "MASK2.BAS", DATE$, TIME$: PRINT #1,
PRINT #1, USING "Number of residues=##"; jmax
FOR j = 1 TO jmax
PRINT #1, USING "    Residue ##      ## building blocks"; j; b(j)
NEXT j
PRINT #1, "
PRINT #1, USING "Number of cells=###"; g: PRINT #1,
FOR j = 2 TO jmax
lmax(j) = lmax(j - 1) * b(j) - 1
w(j) = w(j - 1) / b(j)
NEXT j
FOR j = 1 TO jmax
PRINT #1, USING "Mask for residue ##"; j: PRINT #1,
PRINT #1, USING "    Number of stripes=###"; lmax(j)
PRINT #1, USING "    Width of each stripe=###"; w(j)
FOR i = 1 TO lmax(j)
a = 1 + (i - 1) * w(j) - 1
ac = a + w(j) - 1
PRINT #1, USING "    Stripe ## begins at location ### and ends at ###"; i; a; ac
NEXT i
PRINT #1,
PRINT #1, USING "    For each of ## building blocks, translate mask by ##
cell(s)"; b(j); w(j),
PRINT #1, : PRINT #1, : PRINT #1,
NEXT j

```

© Copyright 1990, Affymax N.V.

35

TABLE 5

Masking Strategy Output

Number of residues= 3

Residue 1 3 building blocks
Residue 2 4 building blocks
Residue 3 5 building blocks

Number of cells= 60

Mask for residue 1

Number of stripes= 1
Width of each stripe= 20
Stripe 1 begins at location 1 and ends at 20
For each of 3 building blocks, translate mask by 20 cell(s)

Mask for residue 2

Number of stripes= 3
Width of each stripe= 5
Stripe 1 begins at location 1 and ends at 5
Stripe 2 begins at location 21 and ends at 25
Stripe 3 begins at location 41 and ends at 45
For each of 4 building blocks, translate mask by 5 cell(s)

Mask for residue 3

Number of stripes= 12
Width of each stripe= 1
Stripe 1 begins at location 1 and ends at 1
Stripe 2 begins at location 6 and ends at 6
Stripe 3 begins at location 11 and ends at 11
Stripe 4 begins at location 16 and ends at 16

TABLE 5-continued

Masking Strategy Output	
Stripe 5 begins at location 21 and ends at 21	
Stripe 6 begins at location 26 and ends at 26	
Stripe 7 begins at location 31 and ends at 31	
Stripe 8 begins at location 36 and ends at 36	
Stripe 9 begins at location 41 and ends at 41	
Stripe 10 begins at location 46 and ends at 46	
Stripe 11 begins at location 51 and ends at 51	
Stripe 12 begins at location 56 and ends at 56	
For each of 5 building blocks, translate mask by 1 cell(s)	

© Copyright 1990, Affymax N.V.

V. Details of One Embodiment of A Fluorescent Detection Device

FIG. 9 illustrates a fluorescent detection device for detecting fluorescently labeled receptors on a substrate. A substrate 112 is placed on an x/y translation table 202. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer 204 which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope 206 which includes one or more objectives 208. Light (about 488 nm) from a laser 210, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror 207 which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror 207 may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope 206 which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter 209 and, thereafter through an aperture plate 211. Wavelength filter 209 may be, for example, a model no. OG530 manufactured by Melles Griot and aperture plate 211 may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube 212 which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter 216. The number of photons is recorded as a function of the location in the computer 204. Pre-Amp 214 may be, for example, a model no. SR440 manufactured by Stanford Research Systems and photon counter 216 may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100 μm with a data collection diameter of about 0.8 to 10 μm preferred. In embodiments with sufficiently high fluorescence, a CCD detector with broad-field illumination is utilized.

By counting the number of photons generated in a given area in response to the laser, it is possible to determine where

15 fluorescent marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescently marked receptor.

20 According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

25 While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

VI. Determination of Relative Binding Strength of Receptors

35 The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

40 In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

45 Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

VII. Examples

50 The following examples are provided to illustrate the efficacy of the inventions herein. All operations were conducted at about ambient temperatures and pressures unless indicated to the contrary.

A. Slide Preparation

55 Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip.

According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from $10^{-7}\%$ to 10% may be used, with about $10^{-3}\%$ to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters (μ l) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500 μ l of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110–120° C. vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500 μ l of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface—that is, those amino groups which have not had the NVOC-GABA attached—are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

B. Synthesis of Eight Trimers of "A" and "B"

FIG. 10 illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitroveratryloxycarbonyl (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photo-reactive at the wavelength of light used to protect the primary chain.

For a monomer set of size n , $n \times l$ cycles are required to synthesize all possible sequences of length l . A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue is to be added, with appropriate washes to remove the by-products of the deprotection.
2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other

embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in FIG. 10A, the glass 20 is provided with regions 22, 24, 26, 28, 30, 32, 34, and 36. Regions 30, 32, 34, and 36 are masked, as shown in FIG. 10B and the glass is irradiated and exposed to a reagent containing "A" (e.g., gly), with the resulting structure shown in FIG. 10C. Thereafter, regions 22, 24, 26, and 28 are masked, the glass is irradiated (as shown in FIG. 10D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in FIG. 10E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in FIG. 10M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

$$n \times l \quad (1)$$

where:

n = the number of monomers in the basis set of monomers, and

l = the number of monomer units in a polymer chain.

Conversely, the synthesized number of sequences of length l will be:

$$n^l \quad (2)$$

Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than l . If, in the extreme case, all polymers having a length less than or equal to l are synthesized, the number of polymers synthesized will be:

$$n^1 + n^{l-1} + \dots + n^l \quad (3)$$

The maximum number of lithographic steps needed will generally be n for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be $n \times l$. The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas} = (A)/(S)$$

where:

A is the total area available for synthesis; and

S is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as a substrate. The durapore membrane was a polyvinylidene difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between -70 and $+50^{\circ}\text{C}$.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standarda and having model no. 824. This kit includes $5.8\text{ }\mu\text{m}$ diameter beads, each impregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in FIG. 9 in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000; 13,000; and 29,000 fluorescein molecules, are shown in FIGS. 11A, 11B, and 11C respectively. On each curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100 μW of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of

photons per fluorescein molecule that can be detected. For the curves illustrated in FIG. 11, this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40–50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then dried and stored in the dark until it was ready to be examined.

Through the use of curves similar to those shown in FIG. 11, and by integrating the fluorescent counts under the exponentially decaying signal, the number of free amino groups on the surface after derivitization was determined. It was determined that slides with labeling densities of 1 fluorescein per $10^3 \times 10^3$ to $\sim 2 \times 2$ nm could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from $10^{-5}\%$ to $10^{-1}\%$.

F. Removal of NVOC and Attachment of a Fluorescent Marker

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

FIG. 12A illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm², ~ 350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in FIG. 12B. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

G. Use of a Mask in Removal of NVOC

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg—Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position versus fluorescence intensity. The fluorescence intensity (in counts) as a function of location is given on the scale to the right of FIG. 13A for a mask having $100 \times 100\text{ }\mu\text{m}$ squares.

The experiment was repeated a number of times through various masks. The fluorescence pattern for a $50\text{ }\mu\text{m}$ mask is illustrated in FIG. 13B, for a $20\text{ }\mu\text{m}$ mask in FIG. 13C, and for a $10\text{ }\mu\text{m}$ mask in FIG. 13D. The mask pattern is distinct down to at least about $10\text{ }\mu\text{m}$ squares using this lithographic technique.

H. Attachment of YGGFL and Subsequent Exposure to Herx Antibody and Goat Antimouse

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be

detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500 μm checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H_2N -tyrosine, glycine, glycine, phenylalanine, leucine- CO_2H , otherwise referred to herein as YGGFL) was attached via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBT/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes at 2 $\mu\text{g}/\text{ml}$ in a supercocktail (containing 1% BSA and 1% ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2 $\mu\text{g}/\text{ml}$ in the supercocktail buffer, and allowed to incubate for 2 hours.

The results of this experiment are provided in FIG. 14. Again, this figure illustrates fluorescence intensity as a function of position. The fluorescence scale is shown on the right. This image was taken at 10 μm steps. This figure indicates that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provides for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide is available for binding with an antibody, and (3) that the detection apparatus capabilities are sufficient to detect binding of a receptor.

I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment and the results thereof are illustrated in FIGS. 15A, 15B, 15C, and 15D.

In FIG. 15A, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated and coupled to NVOC-glycine to form the sequence shown in the last portion of FIG. 15A.

As shown in FIG. 15B, alternating regions of the slide were then illuminated using a projection print using a 500 \times 500 μm checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan is shown in FIG. 15C, and the scale for the fluorescence intensity is again given on the right. Dark areas contain the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluorescein conjugate), and in the red areas YGGFL is present. The YGGFL pentapeptide is recognized by the Herz antibody

and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns are shown for a 50 μm mask used in direct contact ("proximity print") with the substrate in FIG. 15D. Note that the pattern is more distinct and the corners of the checkerboard pattern are touching when the mask is placed in direct contact with the substrate (which reflects the increase in resolution using this technique).

J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL

A synthesis using a 50 μm checkerboard mask similar to that shown in FIG. 15 was conducted. However, P was added to the GGFL sites on the substrate through an additional coupling step. P was added by exposing protected GGFL to light through a mask, and subsequent exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment is provided in FIG. 16. As shown, the regions are again readily discernable. This experiment demonstrates that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

K. Monomer-by-Monomer Synthesis of YGGFL and YPGGFL

In order to further demonstrate the operability of the invention, a 50 μm checkerboard pattern of alternating YGGFL and YPGGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot is provided in FIG. 17. Again, it is seen that the antibody is clearly able to recognize the YGGFL sequence and does not bind significantly at the YPGGFL regions.

L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm \times 0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. FIGS. 18A and 18B illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in FIGS. 18A and 18B were duplicated four times on each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

FIG. 19 is a fluorescence plot of the first slide, which contained only L amino acids. Red indicates strong binding (149,000 counts or more) while black indicates little or no binding of the Herz antibody (20,000 counts or less). The bottom right-hand portion of the slide appears "cut off" because the slide was broken during processing. The sequence YGGFL is clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibit strong recognition of the antibody. By contrast, most of the remaining sequences show little or no binding. The four duplicate portions of the slide are extremely consistent in the amount of binding shown therein.

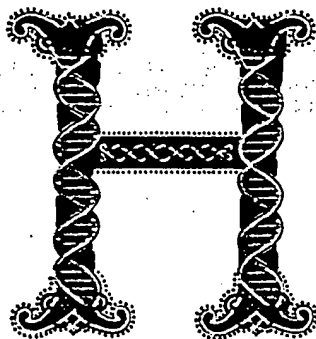
FIG. 20 is a fluorescence plot of the second slide. Again, strongest binding is exhibited by the YGGFL sequence. Significant binding is also detected to YaGFL, YsGFL, and YpGFL. The remaining sequences show less binding with the antibody. Note the low binding efficiency of the sequence yGGFL.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

K

This Page Blank (uspto)

THE HUMAN GENOME



umanity has been given a great gift. With the completion of the human genome sequence, we have received a powerful tool for unlocking the secrets of our genetic heritage and for finding our place among the other participants in the adventure of life.

This week's issue of *Science* contains the report of the sequencing of the human genome from a group of authors led by Craig Venter of Celera Genomics. The report of the sequencing of the human genome from the publicly funded consortium of laboratories led by Francis Collins appears in this week's *Nature*. This stunning achievement has been portrayed—often unfairly—as a competition between two

ventures, one public and one private. That characterization detracts from the awesome accomplishment jointly unveiled this week. In truth, each project contributed to the other. The inspired vision that launched the publicly funded project roughly 10 years ago reflected, and now rewards, the confidence of those who believe that the pursuit of large-scale fundamental problems in the life sciences is in the national interest. The technical innovation and drive of Craig Venter and his colleagues made it possible to celebrate this accomplishment far sooner than was believed possible. Thus, we can salute what has become, in the end, not a contest but a marriage (perhaps encouraged by shotgun) between public funding and private entrepreneurship.

There are excellent scientific reasons for applauding an outcome that has given us two winners. Two sequences are better than one; the opportunity for comparison and convergence is invaluable. Indeed, a real-world proof of the importance of access to both sets of data can be found in the pages of this issue of *Science*, in the comparative analysis by Olivier *et al.* (p. 1298).

Although we have made the point before, it is worth repeating that the sequencing of the human genome represents, not an ending, but the beginning of a new approach to biology. As Galas says in his Viewpoint (p. 1257), the knowledge that all of the genetic components of any process can be identified will give extraordinary new power to scientists. Because of this breakthrough, research can evolve from analyzing the effects of individual genes to a more integrated view that examines whole ensembles of genes as they interact to form a living human being. Several articles in this issue highlight how this approach is already beginning to revolutionize the way we look at human disease.

This has been a massive project, on a scale unparalleled in the history of biology, but of course it has built on the scientific insights of centuries of investigators. By coincidence, this landmark announcement falls during the week of the anniversary of the birth of Charles Darwin. Darwin's message that the survival of a species can depend on its ability to evolve in the face of change is peculiarly pertinent to discussions that have gone on in the past year over access to the Celera data. (Full information regarding the agreements that were reached to make the data available can be found at www.sciencemag.org/feature/data/announcement/gsp.shl.) We are willing to be flexible in allowing data repositories other than the traditional GenBank, while insisting on access to all the data needed to verify conclusions. In this domain, change is everywhere: Commercial researchers are producing more and more potentially valuable sequences, yet (at least in the United States) laws governing databases provide scant protection against piracy. Had the Celera data been kept secret, it would have been a serious loss to the scientific community. We hope that our adaptability in the face of change will enable other proprietary data to be published after peer review, in a way that satisfies our continuing commitment to full access.

It should be no surprise that an achievement so stunning, and so carefully watched, has created new challenges for the scientific venture. *Science* is proud to have played a role in bringing this discovery onto the public stage. It is literally true that this is a historic moment for the scientific endeavor. The human genome has been called the Book of Life. Rather, it is a library, in which, with rules that encourage exploration and reward creativity, we can find many of the books that will help define us and our place in the great tapestry of life.

Barbara R. Jasny and Donald Kennedy

**A historic
moment for
the scientific
endeavor.**

App Serial # 09/854,844
Hu et al.
Novel Human Protease and Polynucleotides Encoding the Same
Exhibit K
LEX-0176-USA

L

This Page Blank (uspto)



US005817479A

United States Patent [19]**Au-Young et al.**[11] **Patent Number:** **5,817,479**[45] **Date of Patent:** **Oct. 6, 1998**[54] **HUMAN KINASE HOMOLOGS**[75] **Inventors:** Janice Au-Young, Berkeley; Olga Bandman; Phillip R. Hawkins, both of Mountain View; Craig G. Wilde, Sunnyvale, all of Calif.[73] **Assignee:** Incyte Pharmaceuticals, Inc., Palo Alto, Calif.[21] **Appl. No.:** **700,575**[22] **Filed:** **Aug. 7, 1996**[51] **Int. Cl.⁶** **C12P 21/06; C12N 15/64**[52] **U.S. Cl.** **435/69.1; 435/91.4; 435/320.1; 435/325; 435/252.1; 536/23.2; 536/23.5**[58] **Field of Search** **536/23.1, 23.2, 536/23.5; 435/91.4, 325, 320.1, 69.1, 252.1**[56] **References Cited****PUBLICATIONS**Taniguchi, "Cytokine Signaling Through Nonreceptor Protein Tyrosine Kinases," *Science*, 268:251-55 (14 Apr. 1995).
Egan et al., "The pathway to signal achievement," *Nature*, 365:781-783.Derijard et al., "Independent Human MAP Kinase Signal Transduction Pathways Defined by MEK and MKK Isoforms," *Science*, 267:682-686 (3 Feb. 1995).R. Davis, "MAPKs: new JNK expands the group," *TIBS*, 19:470-473 (1994).Han et al., "A MAP Kinase Targeted by Endotoxin and Hyperosmolarity in Mammalian Cells," *Science*, 265:808-811 (1994).Levitzi et al., "Tyrosine Kinase Inhibition: An Approach to Drug Development," *Science*, 267:1782-1788 (Mar. 24, 1995).Stroberg, "Functional expression of receptors in microorganisms," *Trends in Pharmacol.*, 13(3)95-98.Hanes et al. *Gen Bank J. Mol. Biol.* 244: 665-672, 1994.Hanes et al. (1994) *J. Mol. Biol.* 244, 665-672.Tamagnone et al. (1994) *Oncogene* 9(12), 3683-3688.Bennett et al. (1994) *J. Biol. Chem.* 269(19), 14211-14218.*Primary Examiner*—Frank C. Eisenschenk*Assistant Examiner*—Patrick J. Nolan*Attorney, Agent, or Firm*—Lucy J. Billings; Sheela Mohan-Peterson; Incyte Pharmaceuticals, Inc.[57] **ABSTRACT**

The present invention provides polynucleotides (kin) which identify and encode novel protein kinases (KIN) expressed in various human cells and tissues. The present invention also provides for antisense sequences and oligonucleotides designed from the nucleotide sequences or their complements. The invention further provides genetically engineered expression vectors and host cells for the production of purified KIN peptides, antibodies capable of binding KIN, and inhibitors specifically bind KIN. The invention specifically provides for diagnostic kits and assays which identify a disorder or disease with altered kinase expression and allow monitoring of patients during drug therapy. These assays utilize oligonucleotides or antibodies produced using the kin polynucleotides.

4 Claims, No Drawings

HUMAN KINASE HOMOLOGS

FIELD OF THE INVENTION

The present invention is in the field of molecular biology; more particularly, the present invention describes nucleic acid sequences for novel human kinase homologs.

BACKGROUND OF THE INVENTION

Kinases regulate many different cell proliferation, differentiation, and signalling processes by adding phosphate groups to proteins. Uncontrolled signalling has been implicated in inflammation, oncogenesis, arteriosclerosis, and psoriasis. Reversible protein phosphorylation is the main strategy for controlling activities of eukaryotic cells. It is estimated that more than 1000 of the 10,000 proteins active in a typical mammalian cell are phosphorylated. The high energy phosphate which drives activation is generally transferred from adenosine triphosphate molecules (ATP) to a particular protein by protein kinases and removed from that protein by protein phosphatases.

Phosphorylation occurs in response to extracellular signals (hormones, neurotransmitters, growth and differentiation factors, etc), cell cycle checkpoints, and environmental or nutritional stresses and is roughly analogous to the turning on a molecular switch. When the switch goes on, the appropriate protein kinase activates a metabolic enzyme, regulatory protein, receptor, cytoskeletal protein, ion channel or pump, or transcription factor.

The kinases comprise the largest known protein family, a superfamily of enzymes with widely varied functions and specificities. They are usually named after their substrate, their regulatory molecules, after some aspect of a mutant phenotype or arbitrarily. Almost all kinases contain a similar 250-300 amino acid catalytic domain. The N-terminal domain, which contains subdomains I-IV, generally folds into a two-lobed structure and binds and orients the ATP (or GTP) donor molecule. The larger C terminal lobe, which contains subdomains VIA-XI, binds the protein substrate and carries out the transfer of the gamma phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Subdomain V spans the two lobes.

The kinases may be categorized into families by the different amino acid sequences (generally between 5 and 100 residues) located on either side of, or inserted into loops of, the kinase domain. These added amino acid sequences allow the regulation of each kinase as it recognizes and interacts with its target protein. The primary structure of the kinase domains is conserved and can be further subdivided into 12 subdomains. The following residues are relatively (~95%) invariant: G₅₀ and G₅₂ in subdomain I, K₇₂ in subdomain II, G₉₁ in subdomain III, E₂₀₈ in subdomain VIII, D₂₂₀ and G₂₂₅ in subdomain IX, and the motifs or patterns of amino acids in subdomains VIB, VIII and IX (Hardie G. and Hanks S. (1995) *The Protein Kinase Facts Books*, I and II, Academic Press, San Diego, Calif.).

The cyclin dependent protein kinase (cdk) family includes proteins which are turned on and off as the cell proceeds through the cell cycle. A cdk is active as a kinase only when it is bound to a cyclin. Cdk activation simultaneously requires both the addition of a high energy phosphate to a threonine residue by a kinase and the removal of a covalently-bound phosphate from a specific tyrosine residue by a phosphatase. The concentration of some cyclins rises gradually through a particular part of the cell cycle until their targeted proteolysis ends the coordinated interaction among the cyclin, kinase, and phosphatase molecules.

The second-messenger dependent protein kinases primarily mediate the effects of second messengers such as cyclic AMP (cAMP) cyclic GMP, inositol triphosphate, phosphatidylinositol, 3,4,5-triphosphate, cyclic ADPrbose, arachidonic acid and diacylglycerol. For purposes of example, the structure and function of cyclic AMP-dependent protein kinase (A-kinase) will be described. Mammalian cells generally contain at least two forms of A-kinase; type 1 which is cytosolic, and type 2 which is bound to plasma membrane, nuclear membrane or microtubules. In its inactive state, A-kinase consists of a complex of two catalytic subunits and two regulatory subunits. When each regulatory subunit has bound two molecules of cAMP, the catalytic subunit is activated and can transfer a high energy phosphate from ATP to the serine or threonine of a substrate protein. Substrate proteins are usually marked by the presence of two or more basic amino acids on their amino terminal sides. A-kinase is important in metabolism of glycogen, for inactivation of phosphatase inhibitor protein, in transcription of genes which contain a regulatory region called the cAMP response element (CRE), and in regulation of the ion channels of olfactory neurons.

Protein kinase C (PKC) is a water-soluble, Ca⁺⁺-dependent kinase, commonly found in brain tissue, which moves to the plasma membrane in the presence of Ca⁺⁺ ions. Approximately half of the known isoforms of PKC are activated initially by diacylglycerol and phosphatidylserine. Prolonged activation of PKC depends on continued production of diacylglycerol molecules which are formed when phospholipases cleave phosphatidylcholine. In nerve cells, PKC phosphorylates ion channels and alters the excitability of the cell membrane. In other cells, activation of PKC increases gene transcription either by triggering a protein kinase cascade which activates a regulatory element (much like CRE above) or by phosphorylating and deactivating an inhibitor of the regulatory protein.

Ca⁺⁺/calmodulin-dependent protein kinases (CaM-kinases) mediate most of the actions of Ca⁺⁺ in human cells. The CaM-kinases include enzymes with narrow substrate specificity such as myosin light chain kinase which activates smooth muscle contraction and phosphorylase kinase which activates glycogen breakdown and the multifunctional enzyme, CaM-kinase II which is found in all cells. Phosphorylase kinase has four subunits: γ is the catalytic moiety and α , β and δ are regulatory. Since subunits α and β are phosphorylated by A-kinase and subunit δ is Ca⁺⁺/calmodulin, glycogen breakdown can be activated by either cAMP or Ca⁺⁺.

CaM-kinase II is particularly enriched in catecholamine synapses. In those neurons, Ca⁺⁺ influx stimulates both the release of dopamine, noradrenaline or adrenaline and also their resynthesis through the activation of CaM-kinase II. Although the main role of CaM-kinase II is phosphorylation of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis, CaM-kinase II also autophosphorylates and remains active until phosphatases overwhelm it.

Transmembrane protein-tyrosine kinases are receptors for most growth factors. The first characterized receptor for epidermal growth factor (EGF) is a single pass transmembrane protein of about 1200 amino acids with an extracellular glycosylated portion that interacts with the 53 amino acid EGF molecule. Binding activates the transfer of a phosphate group from ATP to selected tyrosine side chains of the receptor and other specific proteins. Other protein receptors with similar structure include the following growth and differentiation factors (GF)—platelet derived GF, fibroblast GF, hepatocyte GF, insulin and insulin-like GFs, nerve

GF, vascular endothelial GF, macrophage colony stimulating factor, etc. Each protein phosphorylates itself by receptor dimerization to initiate the intracellular signalling cascade.

Many protein-tyrosine kinases lack transmembrane regions and form a complex with the intercellular regions of other cell surface receptors. The best known NR-PTKs are the Src kinase family (Src, Yes, Fgr, Fyn, Lck, Lyn, Hck, Blk, etc) and the Janus kinase family (Jak1, Jak2, Jak3, Tyk2, etc). The Src PTKs are located on the cytoplasmic side of the plasma membrane and are characterized by Src homology regions 2 and 3 (SH2 and SH3). Src PTKs recognize short peptide motifs bearing phosphotyrosine or proline residues, respectively, and mediate protein-protein interactions that regulate a whole range of intracellular signalling molecules. Janus PTKs contain PTK or PTK-like domains and interact with growth hormone, prolactin, and some of the same cytokine receptors as Src PTKs. The cytokine receptors are unique both in their ability to recruit multiple PTKs and in the diversity of their intracellular domains which allow flexibility in their responses within different cell types (Taniguchi T. (1995) Science 268:251-55). Src and Jak kinases were first identified as the products of mutant oncogenes in cancer cells where their activation was no longer subject to normal cellular controls.

Extracellular signalling proteins such as transforming growth factor- β (TGF- β), activins, bone morphogenetic protein, and related members of the TGF- β superfamily interact with receptor serine/threonine kinases. Like EGF above, these receptor kinases have a single pass transmembrane domain with a serine/threonine kinase residue on the cytosolic side of the plasma membrane. The signalling pathways which are activated by binding the extracellular signalling molecules are presently under investigation.

Mitogen-activated protein (MAP) kinases also regulate intracellular signalling pathways. They mediate signal transduction from cell surface to nuclei via phosphorylation cascades. Several subgroups have been identified, and each manifests different substrate specificities and responds to distinct extracellular stimuli (Egan S. E. and Weinberg R. A. (1993) Nature 365:781-783).

MAP kinase signalling pathways are present in mammalian cells as well as in yeast. The extracellular stimuli which activate mammalian pathways include epidermal growth factor (EGF), ultraviolet light, hyperosmolar medium, heat shock, endotoxin lipopolysaccharide (LPS), and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1). In *Saccharomyces cerevisiae*, exposure to mating pheromone or hyperosmolar environments activate the various MAP kinase signalling pathways.

Mammalian cells have at least three subgroups of MAP kinases (Derijard B. et al (1995) Science 267:682-5), each distinguished by a tripeptide motif. They are extracellular signal-regulated protein kinases (ERK) characterized by Thr-Glu-Tyr; c-Jun amino-terminal kinases (JNK) characterized by Thr-Pro-Tyr; and p38 kinase characterized by Thr-Gly-Tyr. Each subgroup is activated by dual phosphorylation of threonine and tyrosine residues by MAP kinase kinases located upstream of the phosphorylation cascade. Activated MAP kinases, in turn, phosphorylate downstream effectors ultimately leading to intracellular changes.

The ERK signal transduction pathway is activated via tyrosine kinase receptors on the plasmalemma. When growth factors bind to tyrosine, they bind to noncatalytic, Src homology (SH) adaptor proteins (SH2-SH3-SH2) and a guanine nucleotide releasing protein (GNRP). GNRP reduces GTP and activates Ras proteins, members of the

large family of guanine nucleotide binding proteins (G-proteins). Activated Ras proteins bind to a protein kinase C-Raf-1 and activate the Raf-1 proteins. The activated Raf-1 kinase subsequently phosphorylates MAP kinase kinase (MKK) which, in turn, activate ERKs.

ERKs are proline-directed protein kinases which phosphorylate Ser/Thr-Pro motifs. In fact, cytoplasmic phospholipase A2 (cPLA2) and transcription factor Elk-1 are substrates of ERKs. The ERKs phosphorylate Ser₅₀₅ of cPLA2 thereby increasing its enzymatic activity and resulting in release of arachidonic acid and the formation of lysophospholipids from membrane phospholipids. Likewise, phosphorylation of the transcription factor Elk-1 by ERK ultimately increases transcriptional activity.

JNK is distantly related to the ERK and is similarly activated by dual phosphorylation of Thr and Tyr and by MKK4 (Davis R (1994) TIBS 19:470-473). The JNK signal transduction pathway is also initiated by ultraviolet light, osmotic stress, and the pro-inflammatory cytokines, TNF and IL-1. Phosphorylation of Ser₆₃ and Ser₇₃ in the NH₂-terminal domain of the transcription factor c-Jun increases transcriptional activity.

p38 is a 41 kD protein containing 360-amino acids. Its dual phosphorylation is activated by the MKK3 and MKK4, heat shock, hyperosmolar medium, IL-1 or LPS endotoxin (Han J. et al (1994) Science 265:808-811). Sepsis produced by LPS is characterized by fever, chills, tachypnea, and tachycardia, and severe cases may result in septic shock which includes hypotension and multiple organ failure.

Cells respond to LPS as a stress signal because it alters normal cellular processes and induces the release of systemic mediators such as TNF. CD14 is a glycosylphosphatidyl-inositol-anchored membrane glycoprotein which serves as a LPS receptor on the plasmalemma of monocytic cells. The binding of LPS to CD14 causes rapid protein tyrosine phosphorylation of the 44- and 42-/40-kD isoforms of MAP kinases. Although they bind LPS, these MAP kinase isoforms do not appear to belong to the p38 subgroup.

An detailed understanding of kinase pathways and signal transduction is beginning to reveal some mechanisms for interceding in the progression of inflammatory illnesses and of uncontrolled cell proliferation. The cDNAs, oligonucleotides, peptides and antibodies for the human kinases, which are the subject of this invention and are listed in Table 1, provide a plurality of tools for studying signalling cascades in various cells and tissues and for diagnosing and selecting inhibitors or drugs with the potential to intervene in various disorders or diseases in which altered kinase expression is implicated. The disorders or diseases include, but not limited to, human X-linked agammaglobulinemia, nonspherocytic hemolytic anemia, atherosclerosis, carcinomas (breast, ovary, renal, squamous cell and prostate), diabetes, gliomas, glomerular disease, hepatomegaly, Kaposi's sarcoma, lymphoblastic and myelogenous leukemias, myoglobinuria, peptic ulcer disease, psoriasis, pulmonary fibrosis, restenosis, and septic shock due to cholera, *Clostridium difficile*, *E. coli* and *Shigella* (Isselbacher K. J. et al (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, New York City; Levitzki A. and A. Gazit (1995) Science 267:1782-88).

SUMMARY OF THE INVENTION

The subject invention provides unique polynucleotides (SEQ ID NOs 1-44) which have been identified as novel human kinases (kin). These partial cDNAs were identified

among the polynucleotides which comprise various Incyte cDNA libraries.

The invention comprises polynucleotides which are complementary to the kin sequences (SEQ ID Nos 1-44).

The invention also comprises the use of kin sequences to identify and obtain a full length human kinase cDNAs such as SEQ ID NO 45.

The invention further comprises the use of oligomers from these kin sequences in a kinases kit which can be used to identify a disorder or disease with altered kinase expression and provide a method for monitoring progress of a patient during drug therapy.

Aspects of the invention include use of kin sequences or recombinant nucleic acids derived from them to produce purified peptides. Still further aspects of the invention use these purified peptides to identify antibodies or other molecules with inhibitory activity toward a particular kinase, group of kinases or disease.

In addition, the invention comprises the use of kin specific antibodies in assays to identify a disorder or disease with altered kinase expression and provides a method to monitor the progress of a patient during drug therapy.

DESCRIPTION OF THE FIGURE

FIGS. 1A and 1B display the full length nucleotide sequence for human MAP kinase from stomach tissue (SEQ ID NO 45; Incyte Clone 214915E) and its predicted amino acid sequence.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the abbreviation for kinase in lower case (kin) refers to a gene, cDNA, RNA or nucleic acid sequence while the upper case version (KIN) refers to a protein, polypeptide, peptide, oligopeptide, or amino acid sequence.

An "oligonucleotide" or "oligomer" is a stretch of nucleotide residues which has a sufficient number of bases to be used in a polymerase chain reaction (PCR). These short sequences are based on (or designed from) genomic or cDNA sequences and are used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides or oligomers comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 50 nucleotides, preferably about 15 to 30 nucleotides. They are chemically synthesized and may be used as probes.

"Probes" are nucleic acid sequences of variable length, preferably between at least about 10 and as many as about 6,000 nucleotides, depending on use. They are used in the detection of identical, similar, or complementary nucleic acid sequences. Longer length probes are usually obtained from a natural or recombinant source, are highly specific and much slower to hybridize than oligomers. They may be single- or double-stranded and carefully designed to have specificity in PCR, hybridization membrane-based, or ELISA-like technologies.

"Reporter" molecules are chemical moieties used for labelling a nucleic or amino acid sequence. They include, but are not limited to, radionuclides, enzymes, fluorescent, chemi-luminescent, or chromogenic agents. Reporter molecules associate with, establish the presence of, and may allow quantification of a particular nucleic or amino acid sequence.

A "portion" or "fragment" of a polynucleotide or nucleic acid comprises all or any part of the nucleotide sequence

having fewer nucleotides than about 6 kb, preferably fewer than about 1 kb which can be used as a probe. Such probes may be labelled with reporter molecules using nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. After pretesting to optimize reaction conditions and to eliminate false positives, nucleic acid probes may be used in Southern, northern or in situ hybridizations to determine whether DNA or RNA encoding the protein is present in a biological sample, cell type, tissue, organ or organism.

"Recombinant nucleotide variants" are polynucleotides which encode a protein. They may be synthesized by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce specific restriction sites or codon usage-specific mutations, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic host system, respectively.

"Linkers" are synthesized palindromic nucleotide sequences which create internal restriction endonuclease sites for ease of cloning the genetic material of choice into various vectors. "Polylinkers" are engineered to include multiple restriction enzyme sites and provide for the use of both those enzymes which leave 5' and 3' overhangs such as BamHI, EcoRI, PstI, KpnI and Hind III or which provide a blunt end such as EcoRV, SnaBI and StuI.

"Control elements" or "regulatory sequences" are those nontranslated regions of the gene or DNA such as enhancers, promoters, introns and 3' untranslated regions which interact with cellular proteins to carry out replication, transcription, and translation. They may occur as boundary sequences or even split the gene. They function at the molecular level and along with regulatory genes are very important in development, growth, differentiation and aging processes.

"Chimeric" molecules are polynucleotides or polypeptides which are created by combining one or more of nucleotide sequences of this invention (or their parts) with additional nucleic acid sequence(s). Such combined sequences may be introduced into an appropriate vector and expressed to give rise to a chimeric polypeptide which may be expected to be different from the native molecule in one or more of the following kinase characteristics: cellular location, distribution, ligand-binding affinities, interchain affinities, degradation/turnover rate, signalling, etc.

"Active" is that state which is capable of being useful or of carrying out some role. It specifically refers to those forms, fragments, or domains of an amino acid sequence which display the biologic and/or immunogenic activity characteristic of the naturally occurring kinase.

"Naturally occurring KIN" refers to a polypeptide produced by cells which have not been genetically engineered or which have been genetically engineered to produce the same sequence as that naturally produced. Specifically contemplated are various polypeptides which arise from post-translational modifications. Such modifications of the polypeptide include but are not limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

"Derivative" refers to those polypeptides which have been chemically modified by such techniques as ubiquitination, labelling (see above), pegylation (derivatization with polyethylene glycol), and chemical insertion or substitution of amino acids such as ornithine which do not normally occur in human proteins.

"Recombinant polypeptide variant" refers to any polypeptide which differs from naturally occurring KIN by amino acid insertions, deletions and/or substitutions, created using

recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing characteristics of interest may be found by comparing the sequence of KIN with that of related polypeptides and minimizing the number of amino acid sequence changes made in highly conserved regions.

Amino acid "substitutions" are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid "insertions" or "deletions" are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. The variation allowed in a particular amino acid sequence may be experimentally determined by producing the peptide synthetically or by systematically making insertions, deletions, or substitutions of nucleotides in the kin sequence using recombinant DNA techniques.

A "signal or leader sequence" is a short amino acid sequence which or can be used, when desired, to direct the polypeptide through a membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous sources by recombinant DNA techniques.

An "oligopeptide" is a short stretch of amino acid residues and may be expressed from an oligonucleotide. It may be functionally equivalent to and either the same length as or considerably shorter than a "fragment", "portion", or "segment" of a polypeptide. Such sequences comprise a stretch of amino acid residues of at least about 5 amino acids and often about 17 or more amino acids, typically at least about 9 to 13 amino acids, and of sufficient length to display biologic and/or immunogenic activity.

An "inhibitor" is a substance which retards or prevents a chemical or physiological reaction or response. Common inhibitors include but are not limited to antisense molecules, antibodies, antagonists and their derivatives.

A "standard" is a quantitative or qualitative measurement for comparison. Preferably, it is based on a statistically appropriate number of samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles. The samples of a particular standard may be normal or similarly abnormal.

"Animal" as used herein may be defined to include human, domestic (cats, dogs, etc), agricultural (cows, horses, sheep, goats, chicken, fish, etc) or test species (frogs, mice, rats, rabbits, simians, etc).

"Disorders or diseases" in which altered kinase activity have been implicated specifically include, but are not limited to, human X-linked agammaglobulinemia, nonspherocytic hemolytic anemia, atherosclerosis, carcinomas (breast, ovary, renal, squamous cell and prostate), diabetes, gliomas, glomerular disease, hepatomegaly, Kaposi's sarcoma, lymphoblastic and myelogenous leukemias, myoglobinuria, peptic ulcer disease, psoriasis, pulmonary fibrosis, restenosis, and septic shock due to cholera, *Clostridium difficile*, *E. coli* and *Shigella*.

Since the list of technical and scientific terms cannot be all encompassing, any undefined terms shall be construed to have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. Furthermore, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to a "restriction enzyme" or a "high

fidelity enzyme" may include mixtures of such enzymes and any other enzymes fitting the stated criteria, or reference to the method includes reference to one or more methods for obtaining cDNA sequences which will be known to those skilled in the art or will become known to them upon reading this specification.

Before the present sequences, variants, formulations and methods for making and using the invention are described, it is to be understood that the invention is not to be limited only to the particular sequences, variants, formulations or methods described. The sequences, variants, formulations and methodologies may vary, and the terminology used herein is for the purpose of describing particular embodiments. The terminology and definitions are not intended to be limiting since the scope of protection will ultimately depend upon the claims.

DESCRIPTION OF THE INVENTION

The present invention provides for purified partial protein kinase cDNAs which were expressed in various human tissues and isolated therefrom. These sequences were identified by their similarity to published or known open reading frames or untranslated control regions. Since protein kinases are associated with basic cellular processes such as cell proliferation, differentiation and cell signalling, these nucleotide sequences are useful in the characterization of and delineation of normal and abnormal processes. Kinase nucleotide sequences are useful in diagnostic assays used to evaluate the role of a specific kinase in normal, diseased, or therapeutically treated cells.

Purified kinase nucleotide sequences have numerous applications in techniques known to those skilled in the art of molecular biology. These techniques include their use as hybridization probes, for chromosome and gene mapping, in PCR technologies, in the production of sense or antisense nucleic acids, in screening for new therapeutic molecules, etc. These examples are well known and are not intended to be limiting. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including but not limited to such properties as the triplet genetic code and specific base pair interactions.

As a result of the degeneracy of the genetic code, a multitude of kinase-encoding nucleotide sequences may be produced and some of these will bear only minimal homology to the endogenous sequence of any known and naturally occurring kinase. This invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring kinases, and all such variations are to be considered as being specifically disclosed.

Although the kinase nucleotide sequences and their derivatives or variants are preferably capable of identifying the nucleotide sequence of the naturally occurring kinase under optimized conditions, it may be advantageous to produce kinase-encoding nucleotide sequences possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding the kinase without

altering the encoded amino acid sequence include the production of RNA transcripts having more desirable properties, such as a longer half-life, than transcripts produced from the naturally occurring sequence.

Nucleotide sequences encoding a kinase may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (Sambrook J. et al (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; or Ausubel F. M. et al (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York City). Useful sequences for joining to the kinase include an assortment of cloning vectors such as plasmids, cosmids, lambda phage derivatives, phagemids, and the like. Vectors of interest include vectors for replication, expression, probe generation, sequencing, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for one or more host cell systems.

PCR as described in U.S. Pat. Nos. 4,683,195; 4,800,195; and 4,965,188 provides additional uses for oligonucleotides based upon the kinase nucleotide sequence. Such oligomers are generally chemically synthesized, but they may be of recombinant origin or a mixture of both. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'→3') and one with antisense (3' to 5') employed under optimized conditions for identification of a specific gene or diagnostic use. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for identification and/or quantitation of closely related DNA or RNA sequences.

Full length genes may be cloned utilizing partial nucleotide sequence and various methods known in the art. Gobinda et al (1993; *PCR Methods Applic* 2:318-22) disclose "restriction-site PCR" as a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus. First, genomic DNA is amplified in the presence of primer to linker and a primer specific to the known region. The amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase. Gobinda et al present data concerning Factor IX for which they identified a conserved stretch of 20 nucleotides in the 3' noncoding region of the gene.

Inverse PCR is the first method to report successful acquisition of unknown sequences starting with primers based on a known region (Triglia T. et al (1988) *Nucleic Acids Res* 16:8186). The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template. Divergent primers are designed from the known region. The multiple rounds of restriction enzyme digestions and ligations that are necessary prior to PCR make the procedure slow and expensive (Gobinda et al, *supra*).

Capture PCR (Lagerstrom M. et al (1991) *PCR Methods Applic* 1:111-19) is a method for PCR amplification of DNA fragments adjacent to a known sequence in human and YAC DNA. As noted by Gobinda et al (*supra*), capture PCR also requires multiple restriction enzyme digestions and ligations to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before PCR.

Although the restriction and ligation reactions are carried out simultaneously, the requirements for extension, immobilization and two rounds of PCR and purification prior to sequencing render the method cumbersome and time consuming.

Parker J. D. et al (1991; *Nucleic Acids Res* 19:3055-60), teach walking PCR, a method for targeted gene walking which permits retrieval of unknown sequence. Promoter-Finder™ is a new kit available from Clontech (Palo Alto, Calif.) which uses PCR and primers derived from p53 to walk in genomic DNA. Nested primers and special PromoterFinder libraries are used to detect upstream sequences such as promoters and regulatory elements. This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

Another new PCR method, "Improved Method for Obtaining Full Length cDNA Sequences" by Guegler et al, patent application Ser. No 08/487,112, filed Jun. 7, 1995 and hereby incorporated by reference, employs XL-PCR (Perkin-Elmer, Foster City, Calif.) to amplify and extend partial nucleotide sequence into longer pieces of DNA. This method was developed to allow a single researcher to process multiple genes (up to 20 or more) at one time and to obtain an extended (possibly full-length) sequence within 6-10 days. This new method replaces methods which use labelled probes to screen plasmid libraries and allow one researcher to process only about 3-5 genes in 14-40 days.

In the first step, which can be performed in about two days, any two of a plurality of primers are designed and synthesized based on a known partial sequence. In step 2, which takes about six to eight hours, the sequence is extended by PCR amplification of a selected library. Steps 3 and 4, which take about one day, are purification of the amplified cDNA and its ligation into an appropriate vector. Step 5, which takes about one day, involves transforming and growing up host bacteria. In step 6, which takes approximately five hours, PCR is used to screen bacterial clones for extended sequence. The final steps, which take about one day, involve the preparation and sequencing of selected clones.

If the full length cDNA has not been obtained, the entire procedure is repeated using either the original library or some other preferred library. The preferred library may be one that has been size-selected to include only larger cDNAs or may consist of single or combined commercially available libraries, eg. lung, liver, heart and brain from Gibco/BRL (Gaithersburg, Md.). The cDNA library may have been prepared with oligo (dT) or random priming. Random primed libraries are preferred in that they will contain more sequences which contain 5' ends of genes. A randomly primed library may be particularly useful if an oligo (dT) library does not yield a complete gene. It must be noted that the larger and more complex the protein, the less likely it is that the complete gene will be found in a single plasmid.

A new method for analyzing either the size or the nucleotide sequence of PCR products is capillary electrophoresis. Systems for rapid sequencing are available from Perkin Elmer (Foster, City Calif.), Beckman Instruments (Fullerton, Calif.), and other companies. Capillary sequencing employs flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity is converted to electrical signal using appropriate software (eg. Genotyper™ and Sequence Navigators™ from Perkin Elmer) and the entire process from loading of samples to

computer analysis and electronic data display is computer controlled. Capillary electrophoresis provides greater resolution and is many times faster than standard gel based procedures. It is particularly suited to the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample. The reproducible sequencing of up to 350 bp of M13 phage DNA in 30 min has been reported (Ruiz-Martinez M. C. et al (1993) *Anal Chem* 65:2851-8).

Another aspect of the subject invention is to provide for kinase hybridization probes which are capable of hybridizing with naturally occurring nucleotide sequences encoding kinases. The stringency of the hybridization conditions will determine whether the probe identifies only the native nucleotide sequence of that specific kinase or sequences of closely related molecules. If degenerate kinase nucleotide sequences of the subject invention are used for the detection of related kinase encoding sequences, they should preferably contain at least 50% of the nucleotides of the sequences presented herein. Hybridization probes of the subject invention may be derived from the nucleotide sequences of the SEQ ID NOs 1-44, or from surrounding or included genomic sequences comprising untranslated regions such as promoters, enhancers and introns. Such hybridization probes may be labelled with appropriate reporter molecules. Means for producing specific hybridization probes for kinases include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the cDNA sequence may be cloned into a vector for the production of mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labelled nucleotides. A number of companies (such as Pharmacia Biotech, Piscataway, N.J.; Promega, Madison, Wis.; US Biochemical Corp, Cleveland, Ohio; etc.) supply commercial kits and protocols for these procedures.

It is also possible to produce a DNA sequence, or portions thereof, entirely by synthetic chemistry. Sometimes the source of information for producing this sequence comes from the known homologous sequence from closely related organisms. After synthesis, the nucleic acid sequence can be used alone or joined with a preexisting sequence and inserted into one of the many available DNA vectors and their respective host cells using techniques well known in the art. Moreover, synthetic chemistry may be used to introduce specific mutations into the nucleotide sequence. Alternatively, a portion of sequence in which a mutation is desired can be synthesized and recombined with a portion of an existing genomic or recombinant sequence.

The kinase nucleotide sequences can be used individually, or in panels, in a diagnostic test or assay to detect disorder or disease processes associated with abnormal levels of kinase expression. The nucleotide sequence is added to a sample (fluid, cell or tissue) from a patient under hybridizing conditions. After an incubation period, the sample is washed with a compatible fluid which optionally contains a reporter molecule which will bind the specific nucleotide. After the compatible fluid is rinsed off, the reporter molecule is quantitated and compared with a standard for that fluid, cell or tissue. If kinase expression is significantly different from the standard, the assay indicates the presence of disorder or disease. The form of such qualitative or quantitative methods may include northern analysis, dot blot or other membrane based technologies, dip stick, pin or chip technologies, PCR, ELISAs or other multiple sample format technologies.

This same assay, combining a sample with the nucleotide sequence, is applicable in evaluating the efficacy of a

particular therapeutic treatment regime. It may be used in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. First, standard expression must be established for use as a basis of comparison. Second, samples from the animals or patients affected by the disorder or disease are combined with the nucleotide sequence to evaluate the deviation from the standard or normal profile. Third, an existing therapeutic agent is administered, and a treatment profile is generated. The assay is evaluated to determine whether the profile progresses toward or returns to the standard pattern. Successive treatment profiles may be used to show the efficacy of treatment over a period of several days or several months.

The nucleotide sequence for any particular kinase (SEQ ID NOs 1-45) can also be used to generate probes for mapping the native genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. These include in situ hybridization to chromosomal spreads (Verma et al (1988) *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York City), flow-sorted chromosomal preparations, or artificial chromosome constructions such as yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions or single chromosome cDNA libraries.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers are invaluable in extending genetic maps. Examples of genetic maps can be found in the 1994 *Genome Issue of Science* (265:1981f). Often the placement of a gene on the chromosome of another mammalian species may reveal associated markers even if the number or arm of a particular human chromosome is not known. New partial nucleotide sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a disease or syndrome, such as ataxia telangiectasia (AT), has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti et al (1988) *Nature* 336:577-580), any sequences mapping to that area may represent genes for further investigation. The nucleotide sequences of the subject invention may also be used to detect differences in the chromosomal location of nucleotide sequences due to translocation, inversion, etc. between normal and carrier or affected individuals.

The partial nucleotide sequence encoding a particular kinase may be used to produce an amino acid sequence using well known methods of recombinant DNA technology. Goeddel (1990, *Gene Expression Technology, Methods and Enzymology*, Vol 185, Academic Press, San Diego, Calif.) is one among many publications which teach expression of an isolated, purified nucleotide sequence. The amino acid or peptide may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which the nucleotide sequence was derived or from a different species. Advantages of producing an amino acid sequence or peptide by recombinant DNA technology include obtaining adequate amounts for purification and the availability of simplified purification procedures.

Cells transformed with a kinase nucleotide sequence may be cultured under conditions suitable for the expression and recovery of peptide from cell culture. The peptide produced by a recombinant cell may be secreted or may be contained intracellularly depending on the sequence itself and/or the vector used. In general, it is more convenient to prepare

recombinant proteins in secreted form, and this is accomplished by ligating kin to a recombinant nucleotide sequence which directs its movement through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join kin to nucleotide sequence encoding a polypeptide domain which will facilitate protein purification (Kroll D. J. et al (1993) *DNA Cell Biol* 12:441-53).

Direct peptide synthesis using solid-phase techniques (Stewart et al (1969) *Solid-Phase Peptide Synthesis*, WH Freeman Co, San Francisco, Calif.; Merrifield J. (1963) *J Am Chem Soc* 85:2149-2154) is an alternative to recombinant or chimeric peptide production. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer in accordance with the instructions provided by the manufacturer. Additionally a particular kinase sequence or any part thereof may be mutated during direct synthesis and combined using chemical methods with other kinase sequence(s) or a part thereof. This chimeric nucleotide sequence can also be placed in an appropriate vector and host cell to produce a variant peptide.

Although an amino acid sequence or oligopeptide used for antibody induction does not require biological activity, it must be immunogenic. KIN used to induce specific antibodies may have an amino acid sequence consisting of at least five amino acids and preferably at least 10 amino acids. Short stretches of amino acid sequence may be fused with those of another protein such as keyhole limpet hemocyanin, and the chimeric peptide used for antibody production. Alternatively, the oligopeptide may be of sufficient length to contain an entire domain.

Antibodies specific for KIN may be produced by inoculation of an appropriate animal with an antigenic fragment of the peptide. An antibody is specific for KIN if it is produced against an epitope of the polypeptide and binds to at least part of the natural or recombinant protein. Antibody production includes not only the stimulation of an immune response by injection into animals, but also analogous processes such as the production of synthetic antibodies, the screening of recombinant immunoglobulin libraries for specific-binding molecules (Orlandi R. et al (1989) *PNAS* 86:3833-3837, or Huse W. D. et al (1989) *Science* 256:1275-1281), or the in vitro stimulation of lymphocyte populations. Current technology (Winter G. and Milstein C. (1991) *Nature* 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may be adapted to produce molecules which specifically bind kinase peptides. Antibodies or other appropriate molecules generated against a specific immunogenic peptide fragment or oligopeptide can be used in Western analysis, enzyme-linked immunosorbent assays (ELISA) or similar tests to establish the presence of or to quantitate amounts of kinase active in normal, diseased, or therapeutically treated cells or tissues.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

EXAMPLES

I cDNA Library Construction

The kinase sequences of this application (Table 1) were first identified among the sequences comprising various libraries. Technology has advanced considerably since the first cDNA libraries were made. Many small variations in both chemicals and machinery have been instituted over time, and these have improved both the efficiency and safety of the process. Although the cDNAs could be obtained using

an older procedure, the procedure presented in this application is exemplary of one currently being used by persons skilled in the art. For the purpose of providing an exemplary method, the tissue preparation, mRNA isolation and cDNA library construction described here is for the rheumatoid synovium library from which the Incyte Clones 191283 and 192268 for ser/thr kinases were obtained.

Rheumatoid synovial tissue was obtained from the hip joint removed from a 68 year old female with erosive, nodular rheumatoid arthritis. The tissue was frozen, ground to powder in a mortar and pestle, and lysed immediately in buffer containing guanidinium isothiocyanate. The lysate was centrifuged over a CsCl cushion (18 hrs at 25,000 rpm using a Beckman SW28 rotor and ultracentrifuge; Beckman Instruments, Palo Alto, Calif.), ethanol precipitated, resuspended in water and DNase treated for 15 min at 37° C. The RNA was extracted with phenol chloroform and precipitated with ethanol. Polyadenylated messages were isolated using Qiagen Oligotex (QIAGEN Inc, Chatsworth, Calif.), and a custom cDNA library was constructed by Stratagene (La Jolla, Calif.).

First strand cDNA synthesis was accomplished using an oligo (dT) primer/linker which also contained an XhoI restriction site. Second strand synthesis was performed using a combination of DNA polymerase I, *E. coli* ligase and RNase H, followed by the addition of an EcoRI linker to the blunt ended cDNA. The EcoRI linked, double-stranded cDNA was then digested with XhoI restriction enzyme, extracted with phenol chloroform, and fractionated by size on Sephacryl S400. DNA of the appropriate size was then ligated to dephosphorylated Lambda Zap® arms (Stratagene) and packaged using Gigapack extracts (Stratagene). pBluescript (Stratagene) phagemid DNAs were excised en masse from the library.

In the alternative, DNAs were purified using Miniprep Kits (Catalog #77468; Advanced Genetic Technologies Corporation, Gaithersburg, Md.). These kits provide a 96-well format and enough reagents for 960 purifications. The recommended protocol supplied with each kit has been employed except for the following changes. First, the 96 wells are each filled with only 1 ml of sterile Terrific broth (LIFE TECHNOLOGIES™, Gaithersburg, Md.) with carbenicillin at 25 mg/L (2xCarb) and glycerol at 0.4%. After the wells are inoculated, the bacteria are cultured for 24 hours and lysed with 60 µl of lysis buffer. A centrifugation step (2900 rpm for 5 minutes) is performed before the contents of the block are added to the primary filter plate. The optional step of adding isopropanol to TRIS buffer is not routinely performed. After the last step in the protocol, samples are transferred to a Beckman 96-well block for storage.

II Sequencing of cDNA Clones

The cDNA inserts from random isolates of the rheumatoid synovium or other appropriate library were sequenced in part. Methods for DNA sequencing are well known in the art and employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE® (US Biochemical Corp) or Taq polymerase. Methods to extend the DNA from an oligonucleotide primer annealed to the DNA template of interest have been developed for both single- and double-stranded templates. Chain termination reaction products were separated using electrophoresis and detected via their incorporated, labelled precursors. Recent improvements in mechanized reaction preparation, sequencing and analysis have permitted expansion in the number of sequences that can be determined per day. Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200

(Hamilton, Reno, Nev.), Peltier Thermal Cycler (PTC200; MJ Research, Watertown Mass.) and the Applied Biosystems Catalyst 800 and 377 and 373 DNA sequencers.

The quality of any particular cDNA library may be determined by performing a pilot scale analysis of 192 cDNAs and checking for percentages of clones containing vector, lambda or *E. coli* DNA, mitochondrial or repetitive DNA, and clones with exact or homologous matches to public databases. The number of unique sequences—those having no known match in any available database—were recorded.

III Homology Searching of cDNA Clones and Their Deduced Proteins

Each sequence so obtained was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT™ 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (TRW Inc, Los Angeles, Calif.) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT™ 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

Alternatively, BLAST, which stands for Basic Local Alignment Search Tool, is used to search for local sequence alignments (Altschul S. F. (1993) *J Mol Evol* 36:290-300; Altschul, S. F. et al (1990) *J Mol Biol* 215:403-10). BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. While it is useful for matches which do not contain gaps, it is inappropriate for performing motif-style searching. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment BLAST approach is to look threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

All the kinase molecules presented in this application were examined using INHERIT. Although their identification was based on the criteria above, their homology to

known kinase molecules and name are subject to change when additional computer analysis against additional or more recent database information is employed. For example, whereas the first two kinases in Table 1 were initially identified as unique Incyte clones, homologous mouse and human kinases are now known. In other cases, additional sequence information has become available and its review against the known databases has precipitated a name change. Occasionally a clone number will also disappear from the LIFESEQ™ database (Incyte Pharmaceuticals Inc, Palo Alto, Calif.). This situation generally arises during the regular review of clones and assembly of contiguous sequences.

IV Extension of cDNAs to Full Length

The kinase sequences presented here can be used to design oligonucleotide primers for the extension of the cDNAs to full length. In fact, the partial map kinase cDNA sequence (SEQ ID NO 38) initially identified in Incyte clone 214915 among the sequences comprising the human stomach cell library was extended to full length as shown in "A Novel Human Map Kinase Homolog" by Hawkins et al. Incyte Docket PF-036P, filed on Jun. 28, 1995, incorporated herein by reference. The coding region of this full length sequence (SEQ ID NO 45; Incyte Clone 214915E) begins at nucleotide 58 and ends at nucleotide 1156.

Primers are designed based on known sequence; one primer is synthesized to initiate extension in the antisense direction (XLR) and the other to extend sequence in the sense direction (XLF). The primers allow the sequence to be extended "outward" generating amplicons containing new, unknown nucleotide sequence for the gene of interest. The primers may be designed using Oligo 4.0 (National Biosciences Inc, Plymouth, Minn.), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations was avoided.

The stomach cDNA library was used as a template, and XLR=AAG ACA TCC AGG AGC CCA ATG AC and XLF=AGG TGA TCC TCA GCT GGA TGC AC primers were used to extend and amplify the 214915 sequence. By following the instructions for the XL-PCR kit and thoroughly mixing the enzyme and reaction mix, high fidelity amplification is obtained. Beginning with 25 pMol of each primer and the recommended concentrations of all other components of the kit, PCR is performed using the Peltier Thermal Cycler (PTC200; MJ Research, Watertown, Mass.) and the following parameters:

- Step 1 94° C. for 60 sec (initial denaturation)
- Step 2 94° C. for 15 sec
- Step 3 65° C. for 1 min
- Step 4 68° C. for 7 min
- Step 5 Repeat step 2-4 for 15 additional cycles
- Step 6 94° C. for 15 sec
- Step 7 65° C. for 1 min
- Step 8 68° C. for 7 min+15 sec/cycle
- Step 9 Repeat step 6-8 for 11 additional cycles
- Step 10 72° C. for 8 min
- Step 11 4° C. (and holding)

At the end of 28 cycles, 50 μ l of the reaction mix was removed; and the remaining reaction mix was run for an additional 10 cycles as outlined below:

- Step 1 94° C. for 15 sec
- Step 2 65° C. for 1 min

- Step 3 68° C. for (10 min+15 sec)/cycle
 Step 4 Repeat step 1-3 for 9 additional cycles
 Step 5 72° C. for 10 min

A 5-10 μ l aliquot of the reaction mixture is analyzed by electrophoresis on a low concentration (about 0.6-0.8%) agarose mini-gel to determine which reactions were successful in extending the sequence. Although all extensions potentially contain a full length gene, some of the largest products or bands are selected and cut out of the gel. Further purification involves using a commercial gel extraction method such as QIAQuick™ (QIAGEN Inc). After recovery of the DNA, Klenow enzyme is used to trim single-stranded, nucleotide overhangs creating blunt ends which facilitate religation and cloning.

After ethanol precipitation, the products are redissolved in 13 μ l of ligation buffer. Then, 1 μ l T4-DNA ligase (15 units) and 1 μ l T4 polynucleotide kinase are added, and the mixture is incubated at room temperature for 2-3 hours or overnight at 16° C. Competent *E. coli* cells (in 40 μ l of appropriate media) are transformed with 3 μ l of ligation mixture and cultured in 80 μ l of SOC medium (Sambrook J. et al, supra). After incubation for one hour at 37° C., the whole transformation mixture is plated on Luria Bertani (LB)-agar (Sambrook J. et al, supra) containing 2xCarb. The following day, 12 colonies are randomly picked from each plate and cultured in 150 μ l of liquid LB/2xCarb medium placed in an individual well of an appropriate, commercially-available, sterile 96-well microtiter plate. The following day, 5 μ l of each overnight culture is transferred into a non-sterile 96-well plate and after dilution 1:10 with water, 5 μ l of each sample is transferred into a PCR array.

For PCR amplification, 15 μ l of concentrated PCR reaction mix (1.33x) containing 0.75 units of Taq polymerase, a vector primer and one or both of the gene specific primers used for the extension reaction are added to each well. Amplification is performed using the following conditions:

- Step 1 94° C. for 60 sec
 Step 2 94° C. for 20 sec
 Step 3 55° C. for 30 sec
 Step 4 72° C. for 90 sec
 Step 5 Repeat steps 2-4 for an additional 29 cycles
 Step 6 72° C. for 180 sec
 Step 7 4° C. (and holding)

Aliquots of the PCR reactions are run on agarose gels together with molecular weight markers. The sizes of the PCR products are compared to the original partial cDNAs, and appropriate clones are selected, ligated into plasmid and sequenced.

V Diagnostic Assays Using Kinase Specific Oligomers

In those cases where a specific disorder or disease (see definitions supra) is suspected to involve altered quantities of a particular kinase, oligomers may be designed to establish the presence and/or quantity of mRNA expressed in a biological sample. There are several methods currently being used to quantitate the expression of a particular molecule. Most of these methods use radiolabelled (Melby P. C. et al 1993 J Immunol Methods 159:235-44) or biotinylated (Duplaa C. et al 1993 Anal Biochem 229-36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. For example, phosphorylase B kinase deficiency may manifest as hepatomegaly which is inherited as either an X-linked or autosomal recessive trait or myoglobinuria whose inheritance is unknown.

Oligomers for phosphorylase B kinase are first used in quantitative PCR to establish a normal range for expression

of phosphorylase B kinase. Then, these same oligomers are used with extracts of cells from patients with inherited phosphorylase B kinase deficiency. The information from such studies is used to define different inheritance patterns and to diagnose future patients displaying phosphorylase B kinase deficiency-like symptoms. In like manner, this same assay can be used to monitor progress of the patient as his/her physiological situation moves toward the normal range during therapy for the condition.

VI Kinases Kit

The kinases of the subject invention are used to produce a kinases kit for diagnosing disorders or diseases associated with altered kinase expression. This involves the designing a plurality of oligomers, one set of which is specific for each kinase or kinase regulatory sequence. Specificity in this case refers to sequence similarity, to the length of the nucleic acid molecule amplified, to cell or tissue type being screened or to the disorder or disease. These oligomers are combined with a biological sample obtained from a patient in a solution sufficient for PCR and amplified. The PCR products are examined first, to detect the expression of each kinase, and second to quantify the expression of each kinase. Kinase expression is compared with standard ranges for normal and abnormal expression. In the case(s) where kinase expression is altered, use of the kit has provided the physician with a named disorder or disease which can be treated or further investigated.

A further use of the oligomers from the kinases kit is in a diagnostic assay of example V (above) used to monitor patient response to drug therapy. Once the disease has been named and a therapy chosen, the oligomers specific to the patient's disease may be used periodically to monitor the efficacy of the chosen therapy. In this case, the specific oligomers are combined with a biological sample from the patient in a solution sufficient for PCR and amplified. The PCR product is quantified and compared with a normal standard and with the pretreatment profile of the patient. If the kinase expression is tending toward normal, the therapy may be considered effective; if the expression is even more abnormal, therapy should be discontinued and an alternative treatment instituted.

VII Sense or Antisense Molecules

Knowledge of the correct cDNA sequence of any particular kinase, its regulatory elements or parts thereof will enable its use as a tool in sense (Yousoufian H. and H. F. Lodish 1993) Mol Cell Biol 13:98-104) or antisense (Eguchi et al (1991) Annu Rev Biochem 60:631-652) technologies for the investigation of gene function. Oligonucleotides, from genomic or cDNAs, comprising either the sense or the antisense strand of the cDNA sequence can be used in vitro or in vivo to inhibit expression. Such technology is now well known in the art, and oligonucleotides or other fragments can be designed from various locations along the sequences.

The gene of interest can be turned off in the short term by transfecting a cell or tissue with expression vectors which will flood the cell with sense or antisense sequences until all copies of the vector are disabled by endogenous nucleases. Stable transfection of appropriate germ line cells or preferably a zygote with a vector containing the fragment will produce a transgenic organism (U.S. Pat. No. 4,736,866, 12 Apr. 1988), which produces enough copies of the sense or antisense sequence to significantly compromise or entirely eliminate normal activity of the particular kinase gene. Frequently, the function of the gene can be ascertained by observing behaviors such as lethality, loss of a physiological pathway, changes in morphology, etc. at the intracellular, cellular, tissue or organismal level.

In addition to using fragments constructed to interrupt transcription of the open reading frame, modifications of gene expression can be obtained by designing antisense sequences to promoters, enhancers, introns, or even to trans-acting regulatory genes. Similarly, inhibition can be achieved using Hogeboom base-pairing methodology, also known as "triple helix" base pairing.

VIII Expression of Kinases

Expression of the kinases may be accomplished by sub-cloning the cDNAs into appropriate vectors and transfecting the vectors into host cells. In some cases, the cloning vector previously used for the generation of the tissue library also provides for direct expression of kinase sequences in *E. coli*. Upstream of the cloning site, this vector contains a promoter for β -galactosidase, followed by sequence containing the amino-terminal Met and the subsequent 7 residues of β -galactosidase. Immediately following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transfected bacterial strain with IPTG using standard methods will produce a fusion protein corresponding to the first seven residues of β -galactosidase, about 5 to 15 residues which correspond to linker, and the peptide encoded within the kinase cDNA. Since cDNA clone inserts are generated by an essentially random process, there is one chance in three that the included cDNA will lie in the correct frame for proper translation. If the cDNA is not in the proper reading frame, it can be obtained by deletion or insertion of the appropriate number of bases by well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or oligonucleotide linker inclusion.

The kinase cDNA can be shuttled into other vectors known to be useful for expression of protein in specific hosts. Oligonucleotide linkers containing cloning sites as well as a stretch of DNA sufficient to hybridize to the end of the target cDNA (25 bases) can be synthesized chemically by standard methods. These primers can then be used to amplify the desired gene fragments by PCR. The resulting fragments can be digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternatively, similar gene fragments can be produced by digestion of the cDNA with appropriate restriction enzymes and filling in the missing gene sequence with chemically synthesized oligonucleotides. Partial nucleotide sequence from more than one gene can be ligated together and cloned in appropriate vectors to optimize expression.

Suitable expression hosts for such chimeric molecules include but are not limited to mammalian cells such as Chinese Hamster Ovary (CHO) and human 293 cells, insect cells such as Sf9 cells, yeast cells such as *Saccharomyces cerevisiae*, and bacteria such as *E. coli*. For each of these cell systems, a useful expression vector may also include an origin of replication to allow propagation in bacteria and a selectable marker such as the β -lactamase antibiotic resistance gene to allow selection in bacteria. In addition, the vectors may include a second selectable marker such as the neomycin phosphotransferase gene to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts may require RNA processing elements such as 3' polyadenylation sequences if such are not part of the cDNA of interest.

Additionally, some of the kinase vectors may contain native promoters which will allow induction of gene expression in human cells such as the 293 line mentioned above. Other available promoters are host specific and may be specifically combined with the coding region of the kinase

of interest. They include MMTV, SV40, and metallothionein promoters for CHO cells; trp, lac, tac and T7 promoters for bacterial hosts; and alpha factor, alcohol oxidase and PGH promoters for yeast. In addition, transcription enhancers, such as the rous sarcoma virus (RSV) enhancer, may be used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced peptide can be recovered from the conditioned medium and analyzed using methods known in the art.

IX Isolation of Recombinant KIN

KIN may be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, Wash.). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen) between the purification domain and the kin sequence may be useful to facilitate expression of KIN.

X Testing for Kinase Activity

The sequences in this application represent many different domains of different kinase families. These domains (and subdomains as detailed in the background of the invention) may be utilized: 1) individually for the production of antibodies, 2) in functional groups (eg. to span a membrane), and 3) as interchangeable, usable parts of a chimeric kinase. The various partial cDNA sequences of this application represent the different kinase domains of the various families (Hardie G. and Hanks S., supra), and they may be recombined in numerous ways to produce chimeric nucleic acid molecules. For example, a known, full length kinase such as the human map kinase of this application (Seq ID No 45) may be used to swap related portions of the nucleic acid sequence, analogous to domains or subdomains of MAP kinase polypeptides. The chimeric nucleotides, so produced, may be introduced into prokaryotic host cells (as reviewed in Strosberg A. D. and Marullo S. (1992) Trends Pharma Sci 13:95-98) or eukaryotic host cells. These host cells are then employed in procedures to determine what molecules activate the kinase or what molecules are activated by a kinase. Such activating or activated molecules may be of extracellular, intracellular, biologic or chemical origin.

An example of a test system, in this case for protein tyrosine kinases, can be based on the interaction of protein tyrosine kinases with chemokine receptors (Taniguchi T. (1995) Science 268:251-255). These receptors are capable of activating a variety of nonreceptor protein tyrosine kinases when stimulated by an extracellular chemokine. C-X-C chemokines such as platelet factor 4, interleukin-8, connective tissue activating protein III, neutrophil activating peptide 2, are soluble activators of neutrophils.

A standard measure of neutrophil activation involves measuring the mobilization of Ca^{++} as part of the signal transduction pathway. The experiment involves several steps. First, blood cells obtained from venipuncture are fractionated by centrifugation on density gradients. Enriched populations of neutrophils are further fractionated on columns by negative selection using antibodies specific for other blood cells types. Next, neutrophils are transformed with an expression vector containing the kinase nucleic acid sequence of interest and preloaded fluorescent probe whose emission characteristics have been altered by Ca^{++} binding. Or in the alternative, the neutrophil is preloaded with the

purified kinase of interest and fluorescent probe. Then, when the cells are exposed to an appropriate chemokine, the chemokine receptor activates the kinase which, in turn, initiates Ca^{++} flux. Ca^{++} mobilization is observed and measured using fluorometry as has been described in Grynkiewicz G. et al (1985) *J Biol Chem* 260:3440, and McColl S. et al (1993) *J Immunol* 150:4550-4555, incorporated herein by reference.

XI Identification of or Production of Kinase Specific Antibodies

Purified KIN is used to screen a pre-existing antibody library or to raise antibodies using either polyclonal or monoclonal methodology. For polyclonal antibody production, denatured peptide from the reverse phase HPLC separation is obtained in quantities up to 75 mg. This denatured protein can be used to immunize mice or rabbits using standard protocols; about 100 micrograms are adequate for immunization of a mouse, while up to 1 mg might be used to immunize a rabbit. In identifying mouse hybridomas, the denatured protein can be labelled and used to screen potential murine B-cell hybridomas for those which produce antibody. This procedure requires only small quantities of protein, such that 20 mg would be sufficient for labelling and screening of several thousand clones.

For monoclonal antibody production, the amino acid sequence, as deduced from translation of the cDNA, is analyzed to determine regions of high immunogenicity. Peptides comprising appropriate hydrophilic regions are expressed from recombinant cDNA or synthesized and used in suitable immunization protocols to raise antibodies. Selection of appropriate epitopes is described by Ausubel F. M. et al (supra). The optimal amino acid sequences for immunization are usually located at the C-terminus or N-terminus and in intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the protein is in its natural conformation.

Typically, selected oligopeptides, about 15 residues in length, are synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH, Sigma) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel F. M. et al, supra). If necessary, a cysteine may be introduced at the N-terminus of the peptide to permit coupling to KLH. Rabbits are immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity by binding the peptide to plastic, blocking with 1% bovine serum albumin, reacting with antisera, washing and reacting with labelled, affinity purified, specific goat anti-rabbit IgG.

Hybridomas may also be prepared and screened using standard techniques. Hybridomas of interest are detected by screening with labelled KIN to identify those fusions producing the monoclonal antibody with the desired specificity. In a typical protocol, wells of plates (FAST; Becton-Dickinson, Palo Alto, Calif.) are coated during incubation with affinity purified, specific rabbit anti-mouse (or suitable anti-species Ig) antibodies at 10 mg/ml. The coated wells are blocked with 1% BSA, washed and incubated with supernatants from hybridomas. After washing the wells are incubated with labelled KIN at 1 mg/ml. Supernatants with specific antibodies bind more labelled KIN than is detectable in the background. Then clones producing specific antibodies are expanded and subjected to two cycles of cloning at limiting dilution. Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody is purified from mouse ascitic fluid by affinity chromatography on Protein A. Monoclonal antibodies with

affinities of at least $10^8/\text{M}$, preferably 10^9 to 10^{10} or stronger, will typically be made by standard procedures as described in Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; and in Goding (1986) *Monoclonal Antibodies: Principles and Practice*, Academic Press, New York City, both incorporated herein by reference.

XII Diagnostic Assays Using KIN Specific Antibodies

Particular KIN antibodies are useful for investigation of various disorders or diseases which may be characterized by differences in the amount or distribution of KIN. Given the usual role of the kinases, KIN might be expected to be upregulated (or downregulated) in its involvement in activation of signal cascades.

Diagnostic assays for KIN include methods utilizing the antibody and a reporter molecule to detect KIN in human body fluids, membranes, cells, tissues or extracts thereof. The antibodies of the present invention may be used with or without modification. Frequently, the antibodies will be labelled by joining them, either covalently or noncovalently, with a substance which provides for a detectable signal. A wide variety of reporter molecules and conjugation techniques are known and have been reported extensively in both the scientific and patent literature. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemi-luminescent, or chromogenic agents previously mentioned as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immuno-globulins may be produced as shown in U.S. Pat. No. 4,816,567, incorporated herein by reference.

A variety of protocols for measuring soluble or membrane-bound KIN, using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on KIN is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, D. E. et al (1983, *J Exp Med* 158:1211).

XIII Purification of Native KIN Using Antibodies

Native or recombinant protein kinases can be purified by immunoaffinity chromatography using antibodies specific for that particular KIN. In general, an immunoaffinity column is constructed by covalently coupling the anti-KIN antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared from immune sera either by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia Biotech). Likewise, monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified immunoglobulin is covalently attached to a chromatographic resin such as CNBr -activated Sepharose (Pharmacia Biotech). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such immunoaffinity columns may be utilized in the purification of KIN by preparing a fraction from cells containing KIN in a soluble form. This preparation may be derived by solubilization of whole cells or of a subcellular fraction obtained via differential centrifugation (with or without addition of detergent) or by other methods well

known in the art. Alternatively, soluble KIN containing a signal sequence may be secreted in useful quantity into the medium in which the cells are grown.

A soluble KIN-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of KIN (eg, high ionic strength buffers in the presence of detergent). Then, the column is eluted under conditions that disrupt antibody/KIN binding (eg, a buffer of pH 2-3 or a high concentration of a chaotrope such as urea or thiocyanate ion), and KIN is collected.

XIV Drug Screening

This invention is particularly useful for screening therapeutic compounds by using binding fragments of KIN in any of a variety of drug screening techniques. The molecules to be screened may be of extracellular, intracellular, biologic or chemical origin. The peptide fragment employed in such a test may either be free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One may measure, for example, the formation of complexes between KIN and the agent being tested. Alternatively, one can examine the diminution in complex formation between KIN and a receptor caused by the agent being tested.

Methods of screening for drugs or any other agents which can affect signal transduction comprise contacting such an agent with KIN fragment and assaying for the presence of a complex between the agent and the KIN fragment. In such assays, the KIN fragment is typically labelled. After suitable incubation, free KIN fragment is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind to KIN.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the KIN polypeptides and is described in detail in European Patent Application 84/03564, published on Sep. 13, 1984, incorporated herein by reference. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with KIN fragment and washed. Bound KIN fragment is then detected by methods well known in the art. Purified KIN can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding KIN specifically compete with a test compound for binding to KIN fragments. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with KIN.

XV Identification of Molecules Which Interact with KIN

The inventive purified KIN is a research tool for identification, characterization and purification of interacting, signal transduction pathway proteins. Appropriate labels are incorporated into KIN by various methods known in the art and KIN is used to capture soluble or interact with membrane-bound molecules. A preferred method involves labeling the primary amino groups in KIN with ¹²⁵I Bolton-Hunter reagent (Bolton, A. E. and Hunter, W. M. (1973) *Biochem J* 133:529). This reagent has been used to label various molecules without concomitant loss of biological activity (Hebert C. A. et al (1991) *J Biol Chem* 266:18989-94; McColl S. et al (1993) *J Immunol* 150:4550-4555). Membrane-bound molecules are incubated with the labelled KIN molecules, washed to removed unbound molecules, and the KIN complex is quantified. Data obtained using different concentrations of KIN are used to calculate values for the number, affinity, and association of KIN with the signal transduction complex.

Labelled KIN fragments are also useful as a reagent for the purification of molecules with which KIN interacts, specifically including inhibitors. In one embodiment of affinity purification, KIN is covalently coupled to a chromatography column. Cells and their membranes are extracted, KIN is removed and various KIN-free subcomponents are passed over the column. Molecules bind to the column by virtue of their KIN affinity. The KIN-complex is recovered from the column, dissociated and the recovered molecule is subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligomers for cloning its gene from an appropriate cDNA library.

In an alternate method, monoclonal antibodies raised against KIN fragments are screened to identify those which inhibit the binding of labelled KIN. These monoclonal antibodies are then used in affinity purification or expression cloning of associated molecules. Other soluble binding molecules are identified in a similar manner. Labelled KIN is incubated with extracts or other appropriate materials derived from rheumatoid synovium. After incubation, KIN complexes (which are larger than the lone KIN fragment) are identified by a sizing technique such as size exclusion chromatography or density gradient centrifugation and are purified by methods known in the art. The soluble binding protein(s) are subjected to N-terminal sequencing to obtain information sufficient for database identification, if the soluble protein is known, or for cloning, if the soluble protein is unknown.

XVI Use and Administration of Antibodies or Other Inhibitory Molecules

Antibodies, inhibitors, receptors or antagonists of KIN fragments (or other treatments to limit signal transduction, TST), can provide different effects when administered therapeutically. TSTs will be formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although the pH may vary according to the characteristics of the antibody, inhibitor, or antagonist being formulated and the condition to be treated. Characteristics of TSTs include solubility of the molecule, half-life and antigenicity/immunogenicity; these and other characteristics may aid in defining an effective carrier. Native human proteins are preferred as TSTs, but organic or synthetic molecules resulting from drug screens may be equally effective in particular situations.

TSTs may be delivered by known routes of administration including but not limited to topical creams and gels; transmucosal spray and aerosol; transdermal patch and bandage; injectable, intravenous and lavage formulations; and orally administered liquids and pills particularly formulated to resist stomach acid and enzymes. The particular formulation, exact dosage, and route of administration will be determined by the attending physician and will vary according to each specific situation.

Such determinations are made by considering multiple variables such as the condition to be treated, the TST to be administered, and the pharmacokinetic profile of the particular TST. Additional factors which may be taken into account include disease state (e.g. severity) of the patient, age, weight, gender, diet, time and frequency of administration, drug combination, reaction sensitivities, and tolerance/response to therapy. Long acting TST formulations might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular TST.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature. See U.S. Pat. No. 4,657,760; 5,206,344; or 5,225,212. Those

skilled in the art will employ different formulations for different TSTs. Administration to cells such as nerve cells necessitates delivery in a manner different from that to other cells such as vascular endothelial cells.

It is contemplated that disorders or diseases which trigger defensive signal transduction may precipitate damage that is treatable with TSTs. These disorders or diseases may be specifically diagnosed by the tests discussed above, and such testing should be performed in cases where physiologic or pathologic problems are suspected to be associated with abnormal signal transduction.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various

modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

TABLE 1

Clone	Library	GenBank/SwissProt Identifier, Name
297	U937	P00540 Mouse protooncogene <i>ser/thr</i> kinase
1622	U937	HUMCLK3B <i>clk3</i> gene product
10007	THP-1 Phorbol LPS	HSPLK1 protein kinase
12702	THP-1 Phorbol LPS	RATSGPK <i>ser/thr</i> kinase
23789	Inflamed Adenoid	CHKFRNK chicken <i>tyr</i> kinase
35652	HUVEC	KEK5 Chicken <i>Y</i> kinase receptor
35855	HUVEC	HUMANBTK37 <i>tyr</i> kinase
40194	T + B Lymphoblast	KRB1 VARV Variola virus protein kinase
42170	T + B Lymphoblast	HSU09564 serine kinase
46081	Corneal Stroma	YSCKIN1 yeast protein kinase
46651	Corneal Stroma	CDK4, P11802
53840	Fibroblast	HSDAPK, Death-associated protein kinase
54065	Fibroblast	SCPROKIN 1 yeast 35.6 kD
56494	Fibroblast	KLMC RAT, myosin light chain kinase
58029	Skeletal Muscle	ATHCTRIA 1 A. <i>Thaliana</i> <i>Y</i> kinase receptor
64663	Placenta	KIN3 Yeast protein kinase P22209
67967	HUVEC Sheer Stress	YAK1 Yeast protein kinase
68963	HUVEC Sheer Stress	KATK Human <i>Y</i> kinase
71904	Placenta	KIN3 P22209SwP
75289	THP-1 Phorbol	HSU08023 Avian retrovirus <i>rp130</i>
81865	Rheumatoid Synovium	SNF1 Yeast C catabolite derepressing
82056	HUVEC Sheer Stress	P34314 <i>C. elegans</i> <i>ser/thr</i> kinase
108485	AML Blast	KAPA Pig cAMP-dependent protein kinase
114973	Testis	CC2B ARATH Mouse-ear cress <i>cdc</i>
118591	Skeletal Muscle	PBO192 mixed lineage kinase 1
119819	Skeletal Muscle	HSU09564 <i>ser</i> kinase
120376	Skeletal Muscle	U01064 <i>Y</i> kinase
132750	Bone Marrow	MLK2 mixed lineage kinase 2
140052	T Lymphocyte	G-protein coupled receptor kinase
146392	T Lymphocyte	SCYAK1 Yeast <i>Yak1</i> kinase
156108	THP-1 Phorbol LPS	U01064 Dictyostelium <i>Y</i> kinase
173627	Bone Marrow	MMU14166 <i>Kiz</i>
181971	Placenta	HUMTKR <i>Y</i> kinase receptor
182538	Placenta	HSNEK2R kinase
184416*	Cardiac Muscle	KPKS Human proto-oncogene <i>Ser/Thr</i> kinase
191283	Rheumatoid Synovium	RATSGPK <i>Ser/Thr</i> kinase
192268	Rheumatoid Synovium	ATHAPK1A <i>Ser/Thr</i> kinase
214915	Stomach	XLMPK2K Map kinase
223163	Pancreas	TGF- β receptor <i>ser/thr</i> kinase
237002	Small Intestine	P16227 Mouse <i>Y</i> kinase <i>blk</i>
239990	Hippocampus	SHC Human transforming protein
240142	Hippocampus	HSNEK2R
275781	Testes	BOVCK1A casein kinase
285465	Eosinophils	DDIMLCK myosin light chain kinase

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1 1 1) NUMBER OF SEQUENCES: 45

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 526 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v 1) IMMEDIATE SOURCE:

(A) LIBRARY: U937
(B) CLONE: 297

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ACAAGGGTTG TAATTAAAGG CGATTTTGAA ACAATTAAAA TCTGTGATGT AGGAGTCTCT      60
CTACCACTGG ATGAAAATAT GACTGTGACT GACCCTGAGG CTTGTTACAT TGGCACAGAG      120
CCATGGAAGC CCAAAGAAGC TGTGGAGGAG AATGGTGTTA TTAGTGCAAG GCAGACATAT      180
TTGCCTTTGG CTTACTTTGT GGGAAATGAT GACTTTATCG ATTCCACACA TTAATCTTTC      240
AAATGATGAT GATGATGAAG TAAAAACTTT TTGATGAAAA GTAATTTTGA TGTGGAAGCA      300
TTACTATGCA AGCCCTTTGG ACCTAAGGCC ACCCTATTTT AATATTGGAG GACCTTGGTG      360
AATCATACCC AGGAAGGTAA TTTGACCTCT TCTCTGATCA CCCTTATTGA AGCCCCCAAG      420
CACCCTTCTT GTGACAATTT TAGGTTGGAC CAGTTGCTTT GGGCCAACCT AACTAAAGTT      480
GTTCGAAAAA CTTTTTCCA AAAATTTCCA TAGGCCTCCC AAGTTT                        526
```

(2) INFORMATION FOR SEQ ID NO:2:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 378 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v 1) IMMEDIATE SOURCE:

(A) LIBRARY: U937
(B) CLONE: 1622

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
AGAACACCAC ATCCGAGTGG CTGACTTTGG CAGTGCCACA TTTGACCATG AGCACCACAC      60
CACCATTGTG GCCACCCGTC ACTATCGCCG CCTGAGGTGA TCCTTGAGCT GGGCTGGGCA      120
CAGCCTGGTG ACGTCTGGGC ATTGGCTGCA TTCTCTTTGA GTACTACCGG GGCTTCACAC      180
TCTTCCAGAC CCACGAAAAAC CGAGAGCACC TGGTGATGAT GGAGAAGATC CTAGGGCCCCA      240
TCCCATCACA CATGATCCAC CGTACCAGGA AGCAGAATAT TTCTACAAAG GGGGCCTAGT      300
TTGGGATGGA CAGCTCTTAC GGCCGGTATG TAAGGGACTC AAACCTTTAA GGTTCATGTT      360
CAAGCTTCCT GGGAAGTG                        378
```

(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 326 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v 1) IMMEDIATE SOURCE:

(A) LIBRARY: THP-1 Phorbol LPS
(B) CLONE: 10007

(* 1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GGGCTGGCAG CCCGTTTGA GCCTCCGGAG CAGAGGAAGA AGACCATCTT GGCACCCCCA      60
```

-continued-

ACTATGTGGC	TCCAGAAGTG	CTGCTGAGAC	AGGGCCACGG	CCCTGAGGCG	GATGTATGGT	120
CACTGGGCTG	TGTCATGTAC	ACGCTGCTCT	GCGGGACCCT	CCCTTTGAGA	CGGCTGACCT	180
GAAGGAGACG	TACCGCTGCA	TCAAGAAAGT	TCACTACAAC	GGTGCCTGCC	AGCTCTTAAT	240
GCCTGCCCGA	GTCCTTGGCC	GCAATCCTTC	GGGCCTTAAC	CCGAGAACCG	GCCCTCTATT	300
GACAGATCCT	TGCGGCAATT	AACTTT				326

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 257 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: THP-1 Phorbol LPS
- (B) CLONE: 12702

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGCAAGACA	CCTCCTGGAG	GGCCTCCTGA	GAAGGACAGG	CAAAGGGCTG	GGCCAAGGAT	60
GACTTCATGG	AGATTAAGAG	TCATGTTTCT	TCTCCTTAAT	TAAGTGGGAT	GATCTCATT	120
ATAAGAAGAT	TACTCCCCCT	TTTACCCAAA	TGTGAGTGGG	CCCAACGCCT	ACGGACTTTG	180
CCCCGAGTTT	ACGAAGAGCC	TTCCCAATC	CATTGGAAGT	CCCCTGAAAG	GTCCTATACA	240
AGTCAGTTAA	GGAAGTT					257

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 252 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Inflamed Adenoid
- (B) CLONE: 23789

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGAAGAATG	TGGGGCTGAC	CCTCGGAAGT	CATCGGGAGC	GTGGATGATC	TCCTGCCTTC	60
CTTGCCGTCA	TCTACGGAC	AGAGATCGAG	GGCACCCAGA	AACTGCTCAA	CAAAGACCTG	120
GCAGAGCTCA	TCAACAAGAT	GCGCTGGCGC	AAGAACGCGT	GACCTCCCTG	TAGGAGTAAG	180
AGGCAGATCT	GACGGTTTAC	AACCCTGGCT	GTGACGCAAG	AACCTCTTAC	GTGTGCCAGG	240
CCCAAAGTTC	TG					252

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 255 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Huvec
- (B) CLONE: 35652

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

-continued

CAAAATCGTG	GCCCGGAGAA	TGGCGGGGCC	TCAACCCTCT	CCTGGACCAG	CGGCAGCTCA	60
CTACTCAGCT	TTTGGCCTGT	GGGCGAGTGG	CTTCGGGCCA	TCAAAATGGG	AAGATACGAA	120
GAAAGTTTCG	CAGCCGCTGG	CTTTGGCTCC	TTCAGCTGGT	CAGCCAGATC	TCTGCTGAGG	180
ACCTGCTCCG	AATCGAGTCA	CTCTGGCGGG	ACACCAGAAG	AAAATTTGGC	CAGTTCCAGC	240
ACATGAGTCC	CAGGT					255

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 238 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: Huvcc
 (B) CLONE: 35855

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATACCCCA	TATACATAGT	GACTGATATA	TAAGCAATGG	CTGCTTGCTG	AATACCTGAG	60
GAGTCACGGA	AAAGGCTTAA	CCTTCCCAGT	CTTAGAAATG	TGCTACGATG	TCTGTAAGGC	120
ATGGCCTTCT	TGGAGAGTCA	CCAATTCATA	CACCGGGCTT	GGCTGCTCGT	AACTGCTTGG	180
TGGACAGAGA	TCTCTGTGTG	AAAGTTCTCC	ATTTGGATGA	CAAGGTATGT	TCTTGATG	238

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 261 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: T+B Lymphoblast
 (B) CLONE: 40194

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAACAACCTTG	ATTATTTAGG	AATTCCTCTG	TTTTATGGAT	CTGGTCTGAC	TGAATTCAAG	60
GGAAGAAGTT	ACAGATTTAT	GGTAATGGAA	AGACTAGGAA	TAGATTTACA	GAAGATCTCA	120
GGCCAGAATG	GTACCTTTAA	AAAAGTCAACT	GTCCTGCAAT	TAGGATCCGA	ATGTGGATG	180
TACTGGAATA	TATACATGAA	AATGAATATG	TTCATGGTGA	TATAAAAGCA	GCAAATCTAC	240
TTTTGGGTTA	CAAAAATCCT	T				261

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 242 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: T+B Lymphoblast
 (B) CLONE: 42170

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-continued

TAAGAAACCT GAAGATCGAG CCACTGCTGA AGAATGTCTA AAGCACCCCT GGTGACACA	60
GAGCAGTATT CAAGAGCCTT CTTTCAGGAT GGAAAAGGCA CTAGAAGAAG CAAATGCCCT	120
CCAAGAAGGT CATTCTGTGC CTGAAATTAA TTCGGATACC GACAAATCAG AAACCGAGGA	180
ATCCATTGTA ACCGAAGAGT TAATTGTAGT TACTTCATAT ACTCTAGGGC AATGCAGACA	240
GT	242

(2) INFORMATION FOR SEQ ID NO:10:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 222 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

- (v 1.1) IMMEDIATE SOURCE:
 (A) LIBRARY: Corneal Stroma
 (B) CLONE: 46081

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCAAAGGACA GTCCGCCGAG GTGCTCGGTG GAGTCATGGC ATCCCTTTT GGAAGACTGG	60
CCTTGGTGCA AACCTGGAG AAGGTGCCTA TGGAGAAGTT CAACTTGCTG TAAATAGAGT	120
AATAAGAAG CAGTCGCAGT GAAGATTTAG ATATAAGCGT GCCGTAGACT GTCCCGAAAA	180
TATTAAGTAG ATCTGTATCA ATAAAATGCT AATCATGAAA TT	222

(2) INFORMATION FOR SEQ ID NO:11:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 225 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

- (v 1.1) IMMEDIATE SOURCE:
 (A) LIBRARY: Corneal Stroma
 (B) CLONE: 46651

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGCTCCGCC AGTGAGAAGG GCGGCTGCCT GAGCGCCTCA CCAGTCCTCA TCACCCAGAT	60
CCTGTGGCTT TGAGACACCT TCACCTAAGA ACATTTGCCA CTTGACTTAA ACCAGAAACG	120
TGTTTTGTGG CATCAGCAGA CCCTTTCTCA GGTAAGTTGT GCCTTGCTTT TAGCATACGT	180
GAGAAGTTGT TCCGCTCCAT TTTGTGGGAC GTCTTTCTTT CTTTG	225

(2) INFORMATION FOR SEQ ID NO:12:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 256 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

- (v 1.1) IMMEDIATE SOURCE:
 (A) LIBRARY: Fibroblast
 (B) CLONE: 53840

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCGCCTTA CATCTCGCAG CCAAGAACAG CCACCATGAA TGCATCAGGA AGCTGCTTCA	60
TCTAAATGCC CAGCCGAAAG TTTTGACAGC TCTGGGAAAA CAGCTTTACA TTATGCAGCG	120

-continued

GCTCAGGGCT GCCTTCAAGC TGTGCAGATT CTTGCGAACA CAAGAGCCCC ATAAACCTCA 180
 AAGATTTGGA TGGGAATATA CCGCTGCTGC TTGCTGTACA AAATGGTCAC AGTGAGATCT 240
 GTCACITTTT CTGGTC 256

(2) INFORMATION FOR SEQ ID NO:13:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 240 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: cDNA

- (v 1 1) IMMEDIATE SOURCE:
 (A) LIBRARY: Fibroblast
 (B) CLONE: 54065

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTGACATCT GGTCCCTGGG CATATGGCCA TCGAAATGAT TGAAGGGGAG CCTCATACCT 60
 CAATGAAAAC CCTTGAGAGC CTTGTACCTC ATTGCCACCA ATGGGACCCC AGAACTTCAG 120
 AACCAGAGA AGCTGTCAGC TATCTTCCGG GACTTTCTGA ACCGCTGTCT CGAGATGGAT 180
 GTGGAGAAGA GAGGTTGAGC TAAAGAGCTG CTACAGCATC AATTCCTGAA GATTGCCAAT 240

(2) INFORMATION FOR SEQ ID NO:14:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 195 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: cDNA

- (v 1 1) IMMEDIATE SOURCE:
 (A) LIBRARY: Fibroblast
 (B) CLONE: 56494

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACAGTGAAG AGCTCCGAGA AATTATGGGT ACCCTGATAT GTGGCTCCTG AAATTTAGTT 60
 ATGATCCTAT AAGCATGGCA ACAGATATTG GAGCATTGGA GTGTTAACAT ATGTCATGCT 120
 TACAGGAATA TCACCTTTTT AGGCAATGAT AAACAAGAAA CATTCTTAAA CATCTCACAG 180
 ATGATTTTAA GTTAT 195

(2) INFORMATION FOR SEQ ID NO:15:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 207 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: cDNA

- (v 1 1) IMMEDIATE SOURCE:
 (A) LIBRARY: Skeletal Muscle
 (B) CLONE: 58029

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAGTGTTTA TCGAGCCAAA TGGATATCAC AGGACAAGGA GGTGGCTGTA AAGAAGCTCC 60
 TCAAAATAGA GAAAGAGGCA GAAATACTCA GTGTCCTCAG TCACAGAAAC ATCATCCAGT 120
 TTTATGGAGT AATTTTGAAC CTCCCAACTA TGGCATTGTC ACAGAATATG CTTCTTGGGT 180

-continued

CACTCTATGA TTACATTAAC AGTACAA

207

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 184 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: Placenta
 (B) CLONE: 64663

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGGGTGGTA AAAC TTGGAG ATCT TGGGAT TGGCGGTTTT AGCTCAAAAA CCACAGCTGC 60
 ACATTCTTTA GTTGGTACGC CTATTCATGT TCCAGAGGAT ACAGAAATGG ATACAAC TTC 120
 AAATCTCATC TGGTCTCTTG GCTGTCTACT ATATGGATGG CTGCATTACA AAGTCCTTTC 180
 TATG 184

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 206 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: HUVEC Sheer Stress
 (B) CLONE: 67967

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGAATTGCTG AGCATAGACC TTTATGAGCT GATTAAAAAA AATAAGTTTC AGGTTTTAGC 60
 GTCCAGTTGG TACGCAAGTT TGCCCAAGTCC ATCTTGCAAT CTTTGGTGCC CTCCACAAAA 120
 TAAGATTATT CACTGCGATC TGAGCCAGAA AACATTCTCC TGAAACACCA CGGGCGCAGT 180
 TCAACCAAGG TCATTGACTT TGGGTT 206

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 268 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: HUVEC Sheer Stress
 (B) CLONE: 68963

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGAAGTGGC CAGTTTGGAG TGGTCAGCTG GGCAAGTGGA AGGGGCAGTA TGATGTTGCT 60
 GTTAAGATGA TCAAGGAGGG CTCCATGTCA GAAGATGAAT TTTTCAGGAG GCCCAGACTA 120
 TATGAAACTC AGCCATCCCA AGCTGGTTAA ATTCTATGGA GTGTGTTAAA GGATTACCCC 180
 ATATACATGT GACTAATATA TAGCAATGCT TGCTTTTCTG AATTACCTGG GGAGTCACGG 240
 AAAAAGGACT TTTAACCCTT CCCGCTTG 268

-continued

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 224 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: Placenta
 (B) CLONE: 71904

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

CCTGGGGTGG TAAAACTTGG AGACTTGGCT TGGCCGGTTT TCCACCTCAA AAACCACAGC      60
TGCACATCCT TTAGTTGGTA CGCCTTATTA CATGTTCCAG AGAGATACAT GAAAATGGAT      120
ACAACTCAAA CTGACATCTG GCCTTTGGCT GTTACTATAT GAATGGCTGC TTACAAAGCC      180
TTCCTATGGT GACAAAATGA TTTTACTCAT TGTGTAAGAG ATAG                          224

```

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 195 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: THP-1 Phorbol
 (B) CLONE: 75289

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

GCGGGGAATG ACTCCCTATC CTGGGGTCCA GAACCATGAG ATGTATGATA TCTTCTCCAT      60
GGCCACAGGT TGAAGCAGCC CGAAGACTGC CTGGTGAAC TATGAAATA ATGTACTCTT      120
GCTGGAGAAC CGATCCCTTA GACCGCCCA CTTTTTCATA TTGAGGCTGC AGCTAGAAAA      180
ACTCTTAGAA AGTTT                          195

```

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 219 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

- (v i i) IMMEDIATE SOURCE:
 (A) LIBRARY: Rheumatoid Synovium
 (B) CLONE: 81865

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```

CACACGAGAA GCAGAAACAC GACGGGCGGG TAAGATCGGC CACTACATTC TGGTGACACG      60
CTGGGGGTCTG GCACCTTCGG CAAAGTGAAG GTTGGCAAAC ATGATTGACT GGCATAAAGT      120
AGCTGTAAGA TACTCATCGA CAGAAGATTG GGAGCCTTGA TGTGGTAGGA AAAATCCCAG      180
GAAATTCAGA ACCTCAAGCT TTTCAGGCAT CCTCATATA                          219

```

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 181 base pairs
 (B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

(A) LIBRARY: HUVEC Shear Stress
(B) CLONE: 82056

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
CCACCAAAGA TCTCAAATAA AGTTGATGTG TGGTCGGTGG GTGTATCTCT ATCAGTGTCT      60
TTATGGAAGG AAGCCTTTTG GCCATAACCA GTCTCAGCAA GACATCCTAC AAGAGAATAC      120
GATTTTAAAG CTACTGAAGT GCAGTTCCTG CCAAAGCCAG TAGTAACACC TGAAGCAAAG      180
G                                          181
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 218 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

(A) LIBRARY: AML Blast
(B) CLONE: 108485

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
TATGGTTATA TGGAAGAGAA TGTGACTGGT GGTGCGTTGG GGTATTTTTA TACGAAATGC      60
TTGTAGGTGA TACACCTTTT TATGCAGATT CTTTGGTTGG AACTTACAGT AAAATTATGA      120
ACCATAAAAA TTCACCTACC TTTCCTGATG ATAATGACAT ATCAAAAGAA GCAAAAAAACC      180
TTATTTGTGC CTTTCCTTACT GACAGGGAAG TGAGGTTA      218
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 264 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

(A) LIBRARY: Testis
(B) CLONE: 114973

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
GACGGTGGCC ATTTGACATG TGGAGCCTGG GTGCATCACG GTGGAGTTGT ACACGGGCTA      60
CCCCCTGTTT CCCGGGAGAA TGAGGTGGAG CAGCTGGCCT GCATCATGGA GGTGCTGGGT      120
CTGCCGCCAG CCGGCTTCAT TCAGACAGCC TCCAGGAGAC AGACATTCTT TGATTCCAAA      180
GGTTTTCCTA AAAATATAAC CACAACCAGG GGAAAAAAG ATTCCAGATT CCAAGGGCCC      240
TCACGGATTG GTGCTGAAAA AACT                                          264
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 236 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(1) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Skeletal Muscle
- (B) CLONE: 118591

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```

GACTGAGGAC ACTGAAACAT CATCCAGTTT TATGGAGTAA TTCTTGAACC TCCCAACTAT      60
GGCATTGTCA CAGAATATGC TTCTCTGGGA TCACTCTATG ATTACATTAA CAGTAACAGA      120
AGTGAGGAGA TGGATATGGT CACATTATGA CCTGGGCCAC TGATGTAGCC AAAGGAATGC      180
ATTATTTACA TATGGGGCTC CTGTCAAGGT GATTACAGA GACCTCAAGT CAAGGA          236

```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 200 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Skeletal Muscle
- (B) CLONE: 119819

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

CCTGCATGGC CTTGAGCTG GCCACTGGTG ACTACCTGTT CGAGCCGCAT TCTGGAGAAG      60
ACTACAGTCG TGATGAGGGT AAGGGGTGAG GGCTCTGGGC TCAGCCTCCC GGCCTCCCGG      120
CCTGCCTGCC CCAACCTCC TCTTTTGGCC ACAGACCACA TCGCTCACAT AGTGGAGCTT      180
CTGGGGGACA TCCCCCAGC                                     200

```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 217 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Skeletal Muscle
- (B) CLONE: 120376

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```

GATTACAAGT AGCTTGGTTG TAGTGGAAAA AAACGAGAGA TTAACCATTC CAAGCAGTTG      60
CCCCAGAAGT TTTGCTGAAC TTTACATCAG TTTGGGAAGC TGATGCCAAG AAACGGCCAT      120
CATTCAAGCA AATCATTTC AATCCTGGGTC CATGTCAAAT GACACGAGCC TTCCTGCAAG      180
TGTAACATCAT TCCTACACAA CAAGGCGGAG TGGAGGT          217

```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 156 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Bone Marrow
- (B) CLONE: 132750

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```

GTAGATTGGA CTCTGTTGTT TTCTCTCGTA GTTCCCAAAC TCATGGAAGT CTGTTTTTAT      60
CAATATGATG TAAAGTCTGA AATATACAGC TTTGGAATCG TCCTCTGGGA AATCGCCACT      120
GGAGATATCC CGTTTCAAGG CTGTAATTCT GAGAAG                                156

```

(2) INFORMATION FOR SEQ ID NO:29:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: T Lymphocyte
- (B) CLONE: 140052

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```

TGTAATAAG GCCCTTCTCC ACTTGACTTC AGGCAGCAGA TTGTCTAGAA GCCTAAGGAC      60
AGCAATTTCT CTGACAAGAC AAAGTAGATA TTTTATACCA GGGGTTGGCA AACTACTGCC      120
CACGGGCCGA ATTTGGCCCA GTCTGTTTTT GTATGGTGCA AACTAAAAAT GATTTTTTACA      180
TTTTTAAAGA GTTATAAAG AAAAAAATAT GTGGTCTGTG AAAT                        224

```

(2) INFORMATION FOR SEQ ID NO:30:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 198 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: T Lymphocyte
- (B) CLONE: 146392

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

TTTTCTTTGT GTTTTTTTTT GTTCCAGTTT ATTTTAAATG CATATTTTAG TTGATTGCTT      60
TTTTAAAAAG CCCCTCTGG CCTCCTGATT CCAGCTAGTG TCAGCAGTGG GATACCTGCG      120
CTTGAAGGAC ATCATCCACC GTGACATCAA GGATGAGAAC ATCGTGATCG CCGAGGACTT      180
CACAATCAAG CTGATAGT                                198

```

(2) INFORMATION FOR SEQ ID NO:31:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 210 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: THP-1 Phorbol LPS
- (B) CLONE: 156108

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

TGAAAACTAT GAACCTGGAC AAAAATCAAG GGCCAGTATC AAGCACGATA TATATAGCTA      60
TGCAGTTATC ACATGGGAAG TGTTATCCAG AAAACAGCCT TTTGAAGATG TCACCAATCC      120

```

-continued

TTTGACAGATA ATGTATAGTG TGTCACAAGG ACATCGACCT GTTATTAATG AAGAAAGTTT 180
GCCATATGAT ATACCTCACC GAGCACGTAT 210

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 202 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Bone Marrow
- (B) CLONE: 173627

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AGAAGATCGG GGCCGGCTTC TTCTCTGAGG TCTACAAGGT TCGGCACCGA CAGTCAGGGC 60
AAGTATGGTG CTGAAGATGA ACAAGCTCCC CAGTAACCGG GGCAACACAC TACGGGAAGT 120
GCAGCTGATG AACC GGCTCA GGCACCCCAA CATCCTAAGG TTCATGGGAG TCTGTGTGCA 180
CCAGGGACAG CTGCACGCTC TT 202

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 222 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Placenta
- (B) CLONE: 181971

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGTTTTTGGG GGGTTCACAC CTGTCCCTTT CAAATGCTGG CGCTTTCACA CACTCCTTCT 60
CTCCTGCCAG CACCTTCTGG TCTCAGGAGC ATTGCAGGAT GTTGTGTGAG TAAGTATGGG 120
AGACACTTTA GTATGGCTTT TTTAGCTTA GCCTCCTGTT ATCAGAGAGC AGTCTCTTTC 180
AGTGTCAAGG TTTGAGTACT AGATGGTGGA GAAAGCCTGT TT 222

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 192 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Placenta
- (B) CLONE: 182538

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTTGGGGTGG TAAACTTGG AGATCTTGGG CTTGGCCGGT TTTTCAGCTC AAAAACCACA 60
GCTGCACATT CTTAGTTGG TACGCCTTAT TACATGTCTC CAGAGAGAAT ACATGAAAAAT 120
GGATACAACT TCAAATCTGA CATCTGGTCT CTTGGCTGTC TACTATATGA GATGGCTGCA 180
TTACAAAGTC CT 192

-continued

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 152 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Cardiac Muscle
- (B) CLONE: 184416

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```

CTATGGAAGG CCGCTGGCAG GGCAATGACA TTGTCGTGAA GGTGCTGAAG GTTCGAGACT      60
GGAGTACAAG GAAGAGCAGG GACTTCAATG AAGAGTGTCC CCGGCTCAGG ATTTTTCGCA      120
TCCAAATGTG CTCCCAAGTGC TAGGTGCCTG CC                                  152

```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 152 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Rheumatoid Synovium
- (B) CLONE: 191283

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```

CAACTACAGT GAACCTAAAA TGCCTCTAAT ACCTTTGCAA TTATCTTTAA GAGGATATCT      60
TATGAGTGAA ATTAACCTGT GCAACTACTT TCCTATTAC TTTTTCACAG AGACTTAAAA      120
CCAGAGAATA TTTCTAGATT CACAGGGACA CT                                  152

```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 199 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Rheumatoid Synovium
- (B) CLONE: 192268

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

AGTGGACTGC AGTAAGCAGA GCTTCCTGAC CGAGGTGGAG CAGCTGTCCA GGTTCGTCA      60
CCCAAACATT GTGGACTTTC TGGCTACTGT GCTCAGAACG GCTTCTACTG CCTGGTGTAC      120
GGCTTCCTGC CCAACGGCTC CCTGGAGGAC CGTTCCAATG CCAGACCCAG GCCTGCCAC      180
CTCTCTCCTG GCCTCAGCG                                           199

```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 189 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

-continued

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Stomach
- (B) CLONE: 214915

(* i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
AGAAGATCCA GTACCTGGTG TATCAATGCT CAAAGGCCTT AAGTACATCC ACTCTCTGGG      60
GTCGTGCACA GGGACCTGAA GCCAGGCAAC CTGGCTGTGA ATAGGACTGT AACTGAAGAT      120
TCTGGATTTT GGGCTGGCGC GACATGCAGA CGCCGAGATG ACTGGCTACG TGGTGACCCG      180
CTGGTACCT                                     189
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Pancreas
- (B) CLONE: 223163

(* i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
CTTGCTCTTC TGACAGGATG AGAGTTATTA TAAGCAAATC CTACCTAGAG GCTTTTAACT      60
CTAATGGGAA TAACTTGCAA CTAAGAAGACC CAACCTGCAG ACCAAAATTA TCAAATGTTG      120
TGGATTTTCT GTCCCTCTTA ATGGATGTGG TACAATCAGA AAGGTAG                      167
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 197 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Small Intestine
- (B) CLONE: 237002

(* i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
CCCAAACCTG CCCAGCCAGC CCTGAAAATG CAAGTTTTGT ACGATTTTGA AGCTAGGAAC      60
CCACGGGAAC TGA CTGTGGT CCAGGGAGAG AAGCTGGAGG TTTGGACCAC AGCAAGCGGT      120
GGTGGCTGGT GAAGAATAGG CGGGACGGAG CGGCTACATT CCAAGCAACA TCTGGGCCCC      180
TACAGCCGGG GACCCCG                      197
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Hippocampus
- (B) CLONE: 239990

(* i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

-continued

CCAAGATGCT GGAGGAACTC AAGCCGAGAC TTGTACCAAG GAGAGATGAG CAGGAAGGAG	60
GCAGAGGGCT CTGAGAAAGA CGGGACTTCC TGGTCAGGAA GAGCACCACC AACCCGGGCT	120
CCTTTTCCTC ACGGGCATGC ACAATGGCCA GGCAAGCACC TGCTGCTCTT GGACCCAGAA	180
GGCACGTCCG GACAAAAGCA GAGTCTT	207

(2) INFORMATION FOR SEQ ID NO:42:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 195 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

(v 1.1) IMMEDIATE SOURCE:

- (A) LIBRARY: Hippocampus
- (B) CLONE: 240142

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTCACCGGAG AGGATCCATG AGAACGGCTA CAACTTCAAG TCCGACATCT GGTCTTGGG	60
CTGTCTGCTG TACGAGATGG CAGCCCTCCA GAGCCCTTC TATGGAGATA AGATGAATCT	120
TTCTCCCTGT GCCAGAAGAT CGAGCAGTGT GACTACCCCC CACTCCCCGG GGAGCACTAC	180
TCCGAGAAGT TACGT	195

(2) INFORMATION FOR SEQ ID NO:43:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 213 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

(v 1.1) IMMEDIATE SOURCE:

- (A) LIBRARY: Testes
- (B) CLONE: 275781

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTCGTCTATT CGGCACGAGT TTCATTGTCG AAGGAAATAT AAAGTGTCTG GAAGATCTGG	60
TGTAGCTCCT TCGAGACATC TTTGGCGATC AGCATCACCA ACGGTAAGAA GTGTAGTAAG	120
CCAGATCTCA GGGCCAGGCA TCCCCAGTTG CTGTACAAGA GCAGGCTTTC AAGATGCTTC	180
AAGGTCCCTG TCCATCAATA TGCTACACAT TTG	213

(2) INFORMATION FOR SEQ ID NO:44:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 425 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: cDNA

(v 1.1) IMMEDIATE SOURCE:

- (A) LIBRARY: Eosinophils
- (B) CLONE: 285465

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AAATACTTGA AGGAGTTTAT TATCTACATC AGAATAACAT TGTACACCTT GATTTAAAGC	60
CACAGAATAT ATTACTGAGC AGCATATACC CTCTCGGGGA CATTAAAATA GTAGATTTTG	120
GAATGTCTCG AAAAATAGGG CATGCGTGTG AACTTCGGGA AATCATGGGA ACACCAGAAT	180

-continued

ATTTAGCTCC	AGAAATCCTG	AACTATGATC	CCATTACCAC	AGCAACAGAT	ATGTGGAATA	240
TTGGTATAAT	AGCATATATG	TTGTAACTC	ACACATCACC	ATTTGTGGGA	GAAGATAATC	300
AAGAAACATA	CCTCAATATC	TCTCAAGTTA	ATGTAGATTA	TTCGGAAGGA	ACTTTTTTCAT	360
CAGTTTCACA	GCTGGCACAG	ACTTTATTCA	GAGCTTTTAG	TAAAATCAGA	GGAAAAGGCC	420
ACAGC						425

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1851 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(v i i) IMMEDIATE SOURCE:

- (A) LIBRARY: Stomach
- (B) CLONE: 214915E

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCCCCGTGGG	CCGCGAACGC	AGCCGCCACG	CCGGGGCCGC	CGAGATCGGG	TGCCCCGGAT	60
GAGCCTCATC	CGGAAAAAGG	GCTTCTACAA	GCAGGACGTC	AACAAGACCG	CCTGGGAGCT	120
GCCCCAAGACC	TACGTGTCCC	CGACGCACGT	CGGCAGCGGG	GCCTATGGCT	CCGTGTGCTC	180
GGCCATCGAC	AAGCGGTCAG	GGGAGAAGGT	GGCCATCAAG	AAGCTGAGCC	GACCCTTTCA	240
GTCCGAGATC	TTCGCCAAGC	GCGCCTACCG	GGAGCTGCTG	TTGCTGAAAG	ACATGCAGCA	300
TGAGAACGTC	ATTGGGCTCC	TGGATGTCTT	CACCCCAGCC	TCCTCCCTGG	AACTTCTATG	360
ACTTCTACCT	GGTGATGCCC	TTCATGCAGA	CGGATCTGCA	GAAGATCATG	GGGATGGAGT	420
TCAGTGAGGA	GAAGATCCAG	TACCTGGTGT	ATCAGATGCT	CAAAGGCCTT	AAGTACATCC	480
ACTCTGCTGG	GGTCGTGCAC	AGGGACCTGA	AGCCAGGCAA	CCTGGCTGTG	AATGAGGACT	540
GTGAACTGAA	GATTCTGGAT	TTGGGGCTGG	CGCGACATGC	AGACGCCGAG	ATGACTGGCT	600
ACGTGGTGAC	CCGCTGGTAC	CGAGCCCCCG	AGGTGATCCT	CAGCTGGATG	CACTACAACC	660
AGACAGTGGG	CATCTGGTCT	GTGGGCTGTA	TCATGGCAGA	GATGCTGACA	GGGAAAACCTC	720
TGTTCAAGGG	GAAAGATTAC	CTGGACCAGC	TGACCCAGAT	CCTGAAAGTG	ACCGGGGTGC	780
CTGGCACGGA	GTTTGTGCAG	AAGCTGAACG	ACAAAGCGGC	CAAATCCTAC	ATCCAGTCCC	840
TGCCACAGAC	CCCCAGGAAG	GATTTCACTC	AGCTGTTCCC	ACGGGCCAGC	CCCCAGCCTG	900
CGGACCTGCT	GGAGAAGATG	CTGGAGCTAG	ACGTGGACAA	GCGCCTGACG	GCCGCGCAGG	960
CCCTCACCCA	TCCCTTCTTT	GAACCCCTTC	GGGACCCTGA	GGAAGAGACG	GAGGCCCAGC	1020
AGCCGTTTGA	TGATTCTTTA	GAACACGAGA	AACTCACAGT	GGATGAATGG	AAGCAGCACA	1080
TCTACAAAGG	GATTGTGAAC	TTCAGCCCCA	TTGCCCGGAA	GGACTCACGG	CGCCGGAGTG	1140
GCATGAAAGCT	GTAGGGACTC	ATCTTGCATG	GCACCGCCGG	CCAGACACTG	CCCAAGGACC	1200
AGTATTTGTC	ACTACCAAAC	TCAGCCCTTC	TTGGAATACA	GCCTTTCAAG	CAGAGGACAG	1260
AAGGGTCCTT	CTCCTTATGT	GGGAAATGGG	CCTAGTAGAT	GCAGAATTCA	AAGATGTCGG	1320
TTGGGAGAAA	CTAGCTCTGA	TCCTAACAGG	CCACGTTAAA	CTGCCCATCT	GGAGAATCGC	1380
CTGCAGGTGG	GGCCCTTTTC	TTCCCGCCAG	AGTGGGGCTG	AGTGGGGCCT	GAGCCAGGCC	1440
GGGGGCCTAT	GGCAGTGATG	CTGTGTTGGT	TTCTTAGGGA	TGCTCTAACG	AATTACCACA	1500
AACCTGGTGG	ATTGAAACAG	CAGAACTTGA	TTCCCTTACA	GTTCTGGAGG	CTGGAAATCT	1560

-continued

GGGATGGAGG	TGTTGGCAGG	GCTGTGGTCC	CTTTGAAGGC	TCTGGGGAAG	AATCCTTCCT	1620
TGGCTCTTTT	TAGCTTGTGG	CGGCAGTGGG	CAGTCCGTGG	CATTCCCCAG	CTTATTGCTG	1680
CATCACTCCA	GTCTCTGTCT	CTTCTGTTCT	CTCCTCTTTT	AACAACAGTC	ATTGGATTTA	1740
GGGCCCACCC	TAATCCTGTG	TGATCTTATC	TTGATCCTTA	TTAATTAAAC	CTGCAAATAC	1800
TCTAGTTCCA	AATAAAGTCA	CATTCTCAGG	TAAAAAAAAA	AAAAAAAAAA	A	1851

We claim:

1. A purified polynucleotide having a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID

NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, and SEQ ID NO:44.

2. An expression vector comprising the polynucleotide of claim 1.

3. A host cell transformed with the expression vector of claim 2.

4. A method for producing and purifying a polypeptide, said method comprising the steps of:

- a) culturing the host cell of claim 3 under conditions suitable for the expression of the peptide; and
- b) recovering the polypeptide from the host cell culture.

* * * * *

M

This Page Blank (uspto)



US005654173A

United States Patent [19]
Jacobs et al.

[11] **Patent Number:** **5,654,173**
[45] **Date of Patent:** **Aug. 5, 1997**

[54] **SECRETED PROTEINS AND
POLYNUCLEOTIDES ENCODING THEM**

[75] **Inventors:** **Kenneth Jacobs, Newton; John M. McCoy, Reading; Edward R. LaVallie, Tewksbury; Lisa A. Racie; David Merberg, both of Acton; Maurice Treacy, Chestnut Hill; Vikki Spaulding, Billerica, all of Mass.**

[73] **Assignee:** **Genetics Institute, Inc., Cambridge, Mass.**

[21] **Appl. No.:** **702,080**

[22] **Filed:** **Aug. 23, 1996**

[51] **Int. Cl.⁶** **C12P 21/02; C12N 1/21;
C12N 5/10; C07H 21/04**

[52] **U.S. Cl.** **435/69.1; 435/252.3; 435/326;
536/23.5**

[58] **Field of Search** **435/69.1, 326,
435/252.3; 536/23.5**

[56] **References Cited
PUBLICATIONS**

Hunt. Human DNA sequence from cosmid L190B4, Huntington's Disease Region, chromosome 4p 16.3. Direct submission to GenBank, Accession No. Z68276. Dec. 19, 1995.

Sambrook et al. Molecular Cloning: A Laboratory Manual. 2d ed. CSHL Press, Cold Spring Harbor, NY. Chapters 9 and 11. 1989.

Primary Examiner—Vasu S. Jagannathan

Assistant Examiner—Brian Lathrop

Attorney, Agent, or Firm—Scott A. Brown; Thomas J. DesRosier

[57] **ABSTRACT**

Novel polynucleotides and the proteins encoded thereby are disclosed.

14 Claims, No Drawings

App Serial # 09/854,844

Hu et al.

Novel Human Protease and Polynucleotides Encoding the Same

Exhibit M

LEX-0176-USA

SECRETED PROTEINS AND POLYNUCLEOTIDES ENCODING THEM

FIELD OF THE INVENTION

The present invention provides novel polynucleotides and proteins encoded by such polynucleotides, along with therapeutic, diagnostic and research utilities for these polynucleotides and proteins.

BACKGROUND OF THE INVENTION

Technology aimed at the discovery of protein factors (including e.g., cytokines, such as lymphokines, interferons, CSFs and interleukins) has matured rapidly over the past decade. The now routine hybridization cloning and expression cloning techniques clone novel polynucleotides "directly" in the sense that they rely on information directly related to the discovered protein (i.e., partial DNA/amino acid sequence of the protein in the case of hybridization cloning; activity of the protein in the case of expression cloning). More recent "indirect" cloning techniques such as signal sequence cloning, which isolates DNA sequences based on the presence of a now well-recognized secretory leader sequence motif, as well as various PCR-based or low stringency hybridization cloning techniques, have advanced the state of the art by making available large numbers of DNA/amino acid sequences for proteins that are known to have biological activity by virtue of their secreted nature in the case of leader sequence cloning, or by virtue of the cell or tissue source in the case of PCR-based techniques. It is to these proteins and the polynucleotides encoding them that the present invention is directed.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a composition comprising an isolated polynucleotide selected from the group consisting of:

- (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1;
- (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 247 to nucleotide 432;
- (c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 328 to nucleotide 432;
- (d) a polynucleotide comprising the nucleotide sequence of the full length protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146;
- (e) a polynucleotide encoding the full length protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146;
- (f) a polynucleotide comprising the nucleotide sequence of the mature protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146;
- (g) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146;
- (h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:2;
- (i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:2 having biological activity;
- (j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(g) above;

(k) a polynucleotide which encodes a species homologue of the protein of (h) or (i) above.

Preferably, such polynucleotide comprises the nucleotide sequence of SEQ ID NO:1 from nucleotide 247 to nucleotide 432; the nucleotide sequence of SEQ ID NO:1 from nucleotide 328 to nucleotide 432; the nucleotide sequence of the full length protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146; or the nucleotide sequence of the mature protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146. In other preferred embodiments, the polynucleotide encodes the full length or mature protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146.

Other embodiments provide the gene corresponding to the cDNA sequence of SEQ ID NO:1 or SEQ ID NO:3.

In other embodiments, the present invention provides a composition comprising a protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:2;
- (b) fragments of the amino acid sequence of SEQ ID NO:2; and
- (c) the amino acid sequence encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146; the protein being substantially free from other mammalian proteins. Preferably such protein comprises the amino acid sequence of SEQ ID NO:2.

In one embodiment, the present invention provides a composition comprising an isolated polynucleotide selected from the group consisting of:

- (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:4;
- (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:4 from nucleotide 316 to nucleotide 501;
- (c) a polynucleotide comprising the nucleotide sequence of the full length protein, coding sequence of clone BR533_4 deposited under accession number ATCC 98146;
- (d) a polynucleotide encoding the full length protein encoded by the cDNA insert of clone BR533_4 deposited under accession number ATCC 98146;
- (e) a polynucleotide comprising the nucleotide sequence of the mature protein coding sequence of clone BR533_4 deposited under accession number ATCC 98146;
- (f) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone BR533_4 deposited under accession number ATCC 98146;
- (g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:5;
- (h) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:5 having biological activity;
- (i) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(d) above;
- (j) a polynucleotide which encodes a species homologue of the protein of (g) or (h) above.

Preferably, such polynucleotide comprises the nucleotide sequence of SEQ ID NO:4 from nucleotide 316 to nucleotide 501; the nucleotide sequence of the full length protein coding sequence of clone BR533_4 deposited under accession number ATCC 98146; or the nucleotide sequence of the

mature protein coding sequence of clone BR533_4 deposited under accession number ATCC 98146. In other preferred embodiments, the polynucleotide encodes the full length or mature protein encoded by the cDNA insert of clone BR533_4 deposited under accession number ATCC 98146.

Other embodiments provide the gene corresponding to the cDNA sequence of SEQ ID NO:4 or SEQ ID NO:6.

In other embodiments, the present invention provides a composition comprising a protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:5;
- (b) fragments of the amino acid sequence of SEQ ID NO:5; and
- (c) the amino acid sequence encoded by the cDNA insert of clone

BR533_4 deposited under accession number ATCC 98146; the protein being substantially free from other mammalian proteins. Preferably such protein comprises the amino acid sequence of SEQ ID NO:5.

In one embodiment, the present invention provides a composition comprising an isolated polynucleotide selected from the group consisting of:

- (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7;
- (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:7 from nucleotide 113 to nucleotide 433;
- (c) a polynucleotide comprising the nucleotide sequence of the full length protein coding sequence of clone CC288_9 deposited under accession number ATCC 98146;
- (d) a polynucleotide encoding the full length protein encoded by the cDNA insert of clone CC288_9 deposited under accession number ATCC 98146;
- (e) a polynucleotide comprising the nucleotide sequence of the mature protein coding sequence of clone CC288_9 deposited under accession number ATCC 98146;
- (f) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone CC288_9 deposited under accession number ATCC 98146;
- (g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:8;
- (h) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:8 having biological activity;
- (i) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(d) above;
- (j) a polynucleotide which encodes a species homologue of the protein of (g) or (h) above.

Preferably, such polynucleotide comprises the nucleotide sequence of SEQ ID NO:7 from nucleotide 113 to nucleotide 433; the nucleotide sequence of the full length protein coding sequence of clone CC288_9 deposited under accession number ATCC 98146; or the nucleotide sequence of the mature protein coding sequence of clone CC288_9 deposited under accession number ATCC 98146. In other preferred embodiments, the polynucleotide encodes the full length or mature protein encoded by the cDNA insert of clone CC288_9 deposited under accession number ATCC 98146. In yet other preferred embodiments, the present invention provides a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:8 from amino acid 1 to amino acid 77.

Other embodiments provide the gene corresponding to the cDNA sequence of SEQ ID NO:7.

In other embodiments, the present invention provides a composition comprising a protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:8;
- the amino acid sequence of SEQ ID NO:8 from amino acid 1 to amino acid 77;
- (c) fragments of the amino acid sequence of SEQ ID NO:8; and
- (d) the amino acid sequence encoded by the cDNA insert of clone

CC288_9 deposited under accession number ATCC 98146; the protein being substantially free from other mammalian proteins. Preferably such protein comprises the amino acid sequence of SEQ ID NO:8 or the amino acid sequence of SEQ ID NO:8 from amino acid 1 to amino acid 77.

In certain preferred embodiments, the polynucleotide is operably linked to an expression control sequence. The invention also provides a host cell, including bacterial, yeast, insect and mammalian cells, transformed with such polynucleotide compositions.

Processes are also provided for producing a protein, which comprise:

- (a) growing a culture of the host cell transformed with such polynucleotide compositions in a suitable culture medium; and
- (b) purifying the protein from the culture.

The protein produced according to such methods is also provided by the present invention. Preferred embodiments include those in which the protein produced by such process is a mature form of the protein.

Protein compositions of the present invention may further comprise a pharmaceutically acceptable carrier. Compositions comprising an antibody which specifically reacts with such protein are also provided by the present invention.

Methods are also provided for preventing, treating or ameliorating a medical condition which comprises administering to a mammalian subject a therapeutically effective amount of a composition comprising a protein of the present invention and a pharmaceutically acceptable carrier.

DETAILED DESCRIPTION

ISOLATED PROTEINS AND POLYNUCLEOTIDES

Nucleotide and amino acid sequences are reported below for each clone and protein disclosed in the present application. In some instances the sequences are preliminary and may include some incorrect or ambiguous bases or amino acids. The actual nucleotide sequence of each clone can readily be determined by sequencing of the deposited clone in accordance with known methods. The predicted amino acid sequence (both full length and mature) can then be determined from such nucleotide sequence. The amino acid sequence of the protein encoded by a particular clone can also be determined by expression of the clone in a suitable host cell, collecting the protein and determining its sequence.

For each disclosed protein applicants have identified what they have determined to be the reading frame best identifiable with sequence information available at the time of filing. Because of the partial ambiguity in reported sequence information, reported protein sequences include "Xaa" des-

ignators. These "Xaa" designators indicate either (1) a residue which cannot be identified because of nucleotide sequence ambiguity or (2) a stop codon in the determined nucleotide sequence where applicants believe one should not exist (if the nucleotide sequence were determined more accurately).

As used herein a "secreted" protein is one which, when expressed in a suitable host cell, is transported across or through a membrane, including transport as a result of signal sequences in its amino acid sequence. "Secreted" proteins include without limitation proteins secreted wholly (e.g., soluble proteins) or partially (e.g., receptors) from the cell in which they are expressed. "Secreted" proteins also include without limitation proteins which are transported across the membrane of the endoplasmic reticulum.

Clone "BD372_5"

A polynucleotide of the present invention has been identified as clone "BD372_5". BD372_5 was isolated from a human fetal kidney cDNA library using methods which are selective for cDNAs encoding secreted proteins. BD372_5 is a full-length clone, including the entire coding sequence of a secreted protein (also referred to herein as "BD372_5 protein").

The nucleotide sequence of the 5' portion of BD372_5 as presently determined is reported in SEQ ID NO:1. What applicants presently believe is the proper reading frame for the coding region is indicated in SEQ ID NO:2. The predicted acid sequence of the BD372_5 protein corresponding to the foregoing nucleotide sequence is reported in SEQ ID NO:2. Amino acids 1 to 27 are the predicted leader/signal sequence, with the predicted mature amino acid sequence beginning at amino acid 28. Additional nucleotide sequence from the 3' portion of BD372_5, including the polyA tail, is reported in SEQ ID NO:3.

The EcoRI/NotI restriction fragment obtainable from the deposit containing clone BD372_5 should be approximately 2300 bp.

The nucleotide sequence disclosed herein for BD372_5 was searched against the GenBank database using BLASTA/BLASTX and FASTA search protocols. BD372_5 demonstrated at least some identity with ESTs identified as "yc90f12.s 1 Homo sapiens cDNA clone 23278 3'" (R39276, BlastN) and "EST05537 Homo sapiens cDNA clone HFBEM26" (T07647, Fasta). Based upon identity, BD372_5 proteins and each identical protein or peptide may share at least some activity.

Clone "BR533_4"

A polynucleotide of the present invention has been identified as clone "BR533_4". BR533_4 was isolated from a human fetal kidney cDNA library using methods which are selective for cDNAs encoding secreted proteins. BR533_4 is a full-length clone, including the entire coding sequence of a secreted protein (also referred to herein as "BR533_4 protein").

The nucleotide sequence of the 5' portion of BR533_4 as presently determined is reported in SEQ ID NO:4. What applicants presently believe is the proper reading frame for the coding region is indicated in SEQ ID NO:5. The predicted acid sequence of the BR533_4 protein corresponding to the foregoing nucleotide sequence is reported in SEQ ID NO:5. Additional nucleotide sequence from the 3' portion of BR533_4, including the polyA tail, is reported in SEQ ID NO:6.

The EcoRI/NotI restriction fragment obtainable from the deposit containing clone BR533_4 should be approximately 2850 bp.

The nucleotide sequence disclosed herein for BR533_4 was searched against the GenBank database using BLASTA/

BLASTX and FASTA search protocols. BR533_4 demonstrated at least some homology with murine semaphorin E (X85994, BlastN). BR533_4 also shows at least some identity with an EST identified as "yy80d10.s 1 Homo sapiens cDNA clone 279859 3'" (N38844, BlastN). Based upon homology, BR533_4 proteins and each homologous protein or peptide may share at least some activity.

Clone "CC288_9"

A polynucleotide of the present invention has been identified as clone "CC288_9". CC288_9 was isolated from a human adult brain cDNA library using methods which are selective for cDNAs encoding secreted proteins. CC288_9 is a full-length clone, including the entire coding sequence of a secreted protein (also referred to herein as "CC288_9 protein").

The nucleotide sequence of CC288_9 as presently determined is reported in SEQ ID NO:7. What applicants presently believe to be the proper reading frame and the predicted amino acid sequence of the CC288_9 protein corresponding to the foregoing nucleotide sequence is reported in SEQ ID NO:8.

The nucleotide sequence disclosed herein for CC288_9 was searched against the GenBank database using BLASTA/BLASTX and FASTA search protocols. No hits were found in the database.

Deposit of Clones

Clones BD372_5, BR533_4 and CC288_9 were deposited on Aug. 22, 1996 with the American Type Culture Collection under accession number ATCC 98146, from which each clone comprising a particular polynucleotide is obtainable. Each clone has been transfected into separate bacterial cells (*E. coli*) in this composite deposit. Each clone can be removed from the vector in which it was deposited by performing an EcoRI/NotI digestion (5' cite, EcoRI; 3' cite, NotI) to produce the appropriately sized fragment for such clone (approximate clone size fragment are identified below). Bacterial cells containing a particular clone can be obtained from the composite deposit as follows:

An oligonucleotide probe or probes should be designed to the sequence that is known for that particular clone. This sequence can be derived from the sequences provided herein, or from a combination of those sequences. The sequence of the oligonucleotide probe that was used to isolate each full-length clone is identified below, and should be most reliable in isolating the clone of interest.

Clone	Probe Sequence
BD372_5	SEQ ID NO: 9
BR533_4	SEQ ID NO: 10
CC288_9	SEQ ID NO: 11

In the sequences listed above which include an N at position 2, that position is occupied in preferred probes/primers by a biotinylated phosphoramidite residue rather than a nucleotide (such as, for example, that produced by use of biotin phosphoramidite (1-dimethoxytrityloxy-2-(N-biotinyl-4-aminobutyl)-propyl-3-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite) (Glen Research, cat. no. 10-1953)).

The design of the oligonucleotide probe should preferably follow these parameters:

- It should be designed to an area of the sequence which has the fewest ambiguous bases ("N's"), if any;
- It should be designed to have a T_m of approx. 80° C. (assuming 2° for each A or T and 4 degrees for each G or C).

The oligonucleotide should preferably be labeled with γ -³²P ATP (specific activity 6000 Ci/mmol) and T4 polynucle-

otide kinase using commonly employed techniques for labeling oligonucleotides. Other labeling techniques can also be used. Unincorporated label should preferably be removed by gel filtration chromatography or other established methods. The amount of radioactivity incorporated into the probe should be quantitated by measurement in a scintillation counter. Preferably, specific activity of the resulting probe should be approximately 4×10^6 dmp/pmole.

The bacterial culture containing the pool of full-length clones should preferably be thawed and 100 μ l of the stock used to inoculate a sterile culture flask containing 25 ml of sterile L-broth containing ampicillin at 100 μ g/ml. The culture should preferably be grown to saturation at 37° C., and the saturated culture should preferably be diluted in fresh L-broth. Aliquots of these dilutions should preferably be plated to determine the dilution and volume which will yield approximately 5000 distinct and well-separated colonies on solid bacteriological media containing L-broth containing ampicillin at 100 μ g/ml and agar at 1.5% in a 150 mm petri dish when grown overnight at 37° C. Other known methods of obtaining distinct, well-separated colonies can also be employed.

Standard colony hybridization procedures should then be used to transfer the colonies to nitrocellulose filters and lyse, denature and bake them.

The filter is then preferably incubated at 65° C. for 1 hour with gentle agitation in 6 \times SSC (20 \times stock is 175.3 g NaCl/liter, 88.2 g Na citrate/liter, adjusted to pH 7.0 with NaOH) containing 0.5% SDS, 100 μ g/ml of yeast RNA, and 10 mM EDTA (approximately 10 mL per 150 mm filter). Preferably, the probe is then added to the hybridization mix at a concentration greater than or equal to 1×10^6 dpm/mL. The filter is then preferably incubated at 65° C. with gentle agitation overnight. The filter is then preferably washed in 500 mL of 2 \times SSC/0.5% SDS at room temperature without agitation, preferably followed by 500 mL of 2 \times SSC/0.1% SDS at room temperature with gentle shaking for 15 minutes. A third wash with 0.1 \times SSC/0.5% SDS at 65° C. for 30 minutes to 1 hour is optional. The filter is then preferably dried and subjected to autoradiography for sufficient time to visualize the positives on the X-ray film. Other known hybridization methods can also be employed.

The positive colonies are picked, grown in culture, and plasmid DNA isolated using standard procedures. The clones can then be verified by restriction analysis, hybridization analysis, or DNA sequencing.

Fragments of the proteins of the present invention which are capable of exhibiting biological activity are also encompassed by the present invention. Fragments of the protein may be in linear form or they may be cyclized using known methods, for example, as described in H. U. Saragovi, et al., Bio/Technology 10, 773-778 (1992) and in R. S. McDowell, et al., J. Amer. Chem. Soc. 114, 9245-9253 (1992), both of which are incorporated herein by reference. Such fragments may be fused to carrier molecules such as immunoglobulins for many purposes, including increasing the valency of protein binding sites. For example, fragments of the protein may be fused through "linker" sequences to the Fc portion of an immunoglobulin. For a bivalent form of the protein, such a fusion could be to the Fc portion of an IgG molecule. Other immunoglobulin isotypes may also be used to generate such fusions. For example, a protein-IgM fusion would generate a decavalent form of the protein of the invention.

The present invention also provides both full-length and mature forms of the disclosed proteins. The full-length form of the such proteins is identified in the sequence listing by translation of the nucleotide sequence of each disclosed

clone. The mature form of such protein may be obtained by expression of the disclosed full-length polynucleotide (preferably those deposited with ATCC) in a suitable mammalian cell or other host cell. The sequence of the mature form of the protein may also be determinable from the amino acid sequence of the full-length form.

The present invention also provides genes corresponding to the cDNA sequences disclosed herein. The corresponding genes can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include the preparation of probes or primers from the disclosed sequence information for identification and/or amplification of genes in appropriate genomic libraries or other sources of genomic materials.

Where the protein of the present invention is membrane-bound (e.g., is a receptor), the present invention also provides for soluble forms of such protein. In such forms part or all of the intracellular and transmembrane domains of the protein are deleted such that the protein is fully secreted from the cell in which it is expressed. The intracellular and transmembrane domains of proteins of the invention can be identified in accordance with known techniques for determination of such domains from sequence information.

Species homologs of the disclosed polynucleotides and proteins are also provided by the present invention. Species homologs may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source from the desired species.

The invention also encompasses allelic variants of the disclosed polynucleotides or proteins; that is, naturally-occurring alternative forms of the isolated polynucleotide which also encode proteins which are identical, homologous or related to that encoded by the polynucleotides.

The isolated polynucleotide of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means that the isolated polynucleotide of the invention and an expression control sequence are situated within a vector or cell in such a way that the protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the protein. Mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells.

Alternatively, it may be possible to produce the protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins. If the protein is made in yeast or bacteria, it may be necessary to modify the protein

produced therein, for example by phosphorylation or glycosylation of the appropriate sites, in order to obtain the functional protein. Such covalent attachments may be accomplished using known chemical or enzymatic methods.

The protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, Calif., U.S.A. (the MaxBat® kit), and such methods are well known in the art, as described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987), incorporated herein by reference. As used herein, an insect cell capable of expressing a polynucleotide of the present invention is "transformed."

The protein of the invention may be prepared by culturing transformed host cells under culture conditions suitable to express the recombinant protein. The resulting expressed protein may then be purified from such culture (i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography. The purification of the protein may also include an affinity column containing agents which will bind to the protein; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; one or more steps involving hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or immunoaffinity chromatography.

Alternatively, the protein of the invention may also be expressed in a form which will facilitate purification. For example, it may be expressed as a fusion protein, such as those of maltose binding protein (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX). Kits for expression and purification of such fusion proteins are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and In Vitrogen, respectively. The protein can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("Flag") is commercially available from Kodak (New Haven, Conn.).

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The protein thus purified is substantially free of other mammalian proteins and is defined in accordance with the present invention as an "isolated protein."

The protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a nucleotide sequence encoding the protein.

The protein may also be produced by known conventional chemical synthesis. Methods for constructing the proteins of the present invention by synthetic means are known to those skilled in the art. The synthetically-constructed protein sequences, by virtue of sharing primary, secondary or tertiary structural and/or conformational characteristics with proteins may possess biological properties in common therewith, including protein activity. Thus, they may be employed as biologically active or immunological substitutes for natural, purified proteins in screening of therapeutic

compounds and in immunological processes for the development of antibodies.

The proteins provided herein also include proteins characterized by amino acid sequences similar to those of purified proteins but into which modification are naturally provided or deliberately engineered. For example, modifications in the peptide or DNA sequences can be made by those skilled in the art using known techniques. Modifications of interest in the protein sequences may include the alteration, substitution, replacement, insertion or deletion of a selected amino acid residue in the coding sequence. For example, one or more of the cysteine residues may be deleted or replaced with another amino acid to alter the conformation of the molecule. Techniques for such alteration, substitution, replacement, insertion or deletion are well known to those skilled in the art (see, e.g., U.S. Pat. No. 4,518,584). Preferably, such alteration, substitution, replacement, insertion or deletion retains the desired activity of the protein.

Other fragments and derivatives of the sequences of proteins which would be expected to retain protein activity in whole or in part and may thus be useful for screening or other immunological methodologies may also be easily made by those skilled in the art given the disclosures herein. Such modifications are believed to be encompassed by the present invention.

USES AND BIOLOGICAL ACTIVITY

The polynucleotides and proteins of the present invention are expected to exhibit one or more of the uses or biological activities (including those associated with assays cited herein) identified below. Uses or activities described for proteins of the present invention may be provided by administration or use of such proteins or by administration or use of polynucleotides encoding such proteins (such as, for example, in gene therapies or vectors suitable for introduction of DNA).

Research Uses and Utilities

The polynucleotides provided by the present invention can be used by the research community for various purposes. The polynucleotides can be used to express recombinant protein for analysis, characterization or therapeutic use; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides; for selecting and making oligomers for attachment to a "gene chip" or other support, including for examination of expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response. Where the polynucleotide encodes a protein which binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the polynucleotide can also be used in interaction trap assays (such as, for example, that described in Gyuris et al., *Cell* 75:791-803 (1993)) to identify polynucleotides encoding the other protein with which binding occurs or to identify inhibitors of the binding interaction.

The proteins provided by the present invention can similarly be used in assay to determine biological activity, including in a panel of multiple proteins for high-throughput screening; to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands. Where the protein binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

Nutritional Uses

Polynucleotides and proteins of the present invention can also be used as nutritional sources or supplements. Such uses include without limitation use as a protein or amino acid supplement, use as a carbon source, use as a nitrogen source and use as a source of carbohydrate. In such cases the protein or polynucleotide of the invention can be added to the feed of a particular organism or can be administered as a separate solid or liquid preparation, such as in the form of powder, pills, solutions, suspensions or capsules. In the case of microorganisms, the protein or polynucleotide of the invention can be added to the medium in or on which the microorganism is cultured.

Cytokine and Cell Proliferation/Differentiation Activity

A protein of the present invention may exhibit cytokine, cell proliferation (either inducing or inhibiting) or cell differentiation (either inducing or inhibiting) activity or may induce production of other cytokines in certain cell populations. Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity. The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines including, without limitation, 32D, DA2, DA1G, T10, B9, B9/11, BaF3, MC9/G, M+(preB M+), 2E8, RB5, DA1, 123, T1165, HT2, CTLL2, TF-1, Mo7c and CMK.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for T-cell or thymocyte proliferation include without limitation those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., *J. Immunol.* 137:3494-3500, 1986; Bertagnolli et al., *J. Immunol.* 145:1706-1712, 1990; Bertagnolli et al., *Cellular Immunology* 133:327-341, 1991; Bertagnolli, et al., *J. Immunol.* 149:3778-3783, 1992; Bowman et al., *J. Immunol.* 152:1756-1761, 1994.

Assays for cytokine production and/or proliferation of spleen cells, lymph node cells or thymocytes include, without limitation, those described in: *Polyclonal T cell stimulation*, Kruisbeek, A. M. and Shevach, E. M. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 3.12.1-3.12.14, John Wiley and Sons, Toronto, 1994; and *Measurement of mouse and human interleukin γ* , Schreiber, R. D. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 6.8.1-6.8.8, John Wiley and Sons, Toronto, 1994.

Assays for proliferation and differentiation of hematopoietic and lymphopoietic cells include, without limitation, those described in: *Measurement of Human and Murine Interleukin 2 and Interleukin 4*, Bottomly, K., Davis, L. S. and Lipsky, P. E. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 6.3.1-6.3.12, John Wiley and Sons, Toronto, 1991; deVries et al., *J. Exp. Med.* 173:1205-1211, 1991; Moreau et al., *Nature* 336:690-692, 1988; Greenberger et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2931-2938, 1983; *Measurement of mouse and human interleukin 6*—Nordan, R. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 6.6.1-6.6.5, John Wiley and Sons, Toronto, 1991; Smith et al., *Proc. Natl. Acad. Sci. U.S.A.* 83:1857-1861, 1986; *Measurement of human Interleukin 11*—Bennett, F., Giannotti, J., Clark, S. C. and Turner, K. J. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 6.15.1 John Wiley and Sons, Toronto, 1991; *Measurement of mouse and human Interleukin 9*—Ciarletta, A., Giannotti, J., Clark, S. C. and Turner, K. J. In *Current Protocols in Immunology*, J. E. e.a. Coligan eds. Vol 1 pp. 6.13.1, John Wiley and Sons, Toronto, 1991.

Assays for T-cell clone responses to antigens (which will identify, among others, proteins that affect APC-T cell interactions as well as direct T-cell effects by measuring proliferation and cytokine production) include, without limitation, those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function; Chapter 6, Cytokines and their cellular receptors; Chapter 7, Immunologic studies in Humans); Weinberger et al., *Proc. Natl. Acad. Sci. USA* 77:6091-6095, 1980; Weinberger et al., *Eur. J. Immunol.* 11:405-411, 1981; Takai et al., *J. Immunol.* 137:3494-3500, 1986; Takai et al., *J. Immunol.* 140:508-512, 1988.

Immune Stimulating or Suppressing Activity
A protein of the present invention may also exhibit immune stimulating or immune suppressing activity, including without limitation the activities for which assays are described herein. A protein may be useful in the treatment of various immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating (up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations. These immune deficiencies may be genetic or be caused by viral (e.g., HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders. More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using a protein of the present invention, including infections by HIV, hepatitis viruses, herpesviruses, mycobacteria, *Leishmania* spp., malaria spp. and various fungal infections such as candidiasis. Of course, in this

regard, a protein of the present invention may also be useful where a boost to the immune system generally may be desirable, i.e., in the treatment of cancer.

Autoimmune disorders which may be treated using a protein of the present invention include, for example, connective tissue disease, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes mellitus, myasthenia gravis, graft-versus-host disease and autoimmune inflammatory eye disease. Such a protein of the present invention may also be useful in the treatment of allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems. Other conditions, in which immune suppression is desired (including, for example, organ transplantation), may also be treatable using a protein of the present invention.

Using the proteins of the invention it may also be possible to immune responses, in a number of ways. Down regulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T cells may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent.

Down regulating or preventing one or more antigen functions (including without limitation B lymphocyte antigen functions (such as, for example, B7)), e.g., preventing high level lymphokine synthesis by activated T cells, will be useful in situations of tissue, skin and organ transplantation and in graft-versus-host disease (GVHD). For example, blockage of T cell function should result in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 lymphocyte antigen with its natural ligand(s) on immune cells (such as a soluble, monomeric form of a peptide having B7-2 activity alone or in conjunction with a monomeric form of a peptide having an activity of another B lymphocyte antigen (e.g., B7-1, B7-3) or blocking antibody), prior to transplantation can lead to the binding of the molecule to the natural ligand(s) on the immune cells without transmitting the corresponding costimulatory signal. Blocking B lymphocyte antigen function in this manner prevents cytokine synthesis by immune cells, such as T cells, and thus acts as an immunosuppressant. Moreover, the lack of costimulation may also be sufficient to anergize the T cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by B lymphocyte antigen-blocking reagents may avoid the necessity of repeated administration of these blocking reagents. To achieve sufficient immunosuppression or tolerance in a subject, it may also be necessary to block the function of a combination of B lymphocyte antigens.

The efficacy of particular blocking reagents in preventing organ transplant rejection or GVHD can be assessed using animal models that are predictive of efficacy in humans. Examples of appropriate systems which can be used include

allogeneic cardiac grafts in rats and xenogeneic pancreatic islet cell grafts in mice, both of which have been used to examine the immunosuppressive effects of CTLA4Ig fusion proteins in vivo as described in Lenschow et al., *Science* 257:789-792 (1992) and Turka et al., *Proc. Natl. Acad. Sci. USA*, 89:11102-11105 (1992). In addition, murine models of GVHD (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 846-847) can be used to determine the effect of blocking B lymphocyte antigen function in vivo on the development of that disease.

Blocking antigen function may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of T cells by disrupting receptor/ligand interactions of B lymphocyte antigens can be used to inhibit T cell activation and prevent production of autoantibodies or T cell-derived cytokines which may be involved in the disease process. Additionally, blocking reagents may induce antigen-specific tolerance of autoreactive T cells which could lead to long-term relief from the disease. The efficacy of blocking reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in MRL/lpr/lpr mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

Upregulation of an antigen function (preferably a B lymphocyte antigen function), as a means of up regulating immune responses, may also be useful in therapy. Upregulation of immune responses may be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through stimulating B lymphocyte antigen function may be useful in cases of viral infection. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of stimulatory forms of B lymphocyte antigens systemically.

Alternatively, anti-vital immune responses may be enhanced in an infected patient by removing T cells from the patient, costimulating the T cells in vitro with viral antigen-pulsed APCs either expressing a peptide of the present invention or together with a stimulatory form of a soluble peptide of the present invention and reintroducing the in vitro activated T cells into the patient. Another method of enhancing anti-viral immune responses would be to isolate infected cells from a patient, transfect them with a nucleic acid encoding a protein of the present invention as described herein such that the cells express all or a portion of the protein on their surface, and reintroduce the transfected cells into the patient. The infected cells would now be capable of delivering a costimulatory signal to, and thereby activate, T cells in vivo.

In another application, up regulation or enhancement of antigen function (preferably B lymphocyte antigen function) may be useful in the induction of tumor immunity. Tumor cells (e.g., sarcoma, melanoma, lymphoma, leukemia, neuroblastoma, carcinoma) transfected with a nucleic acid encoding at least one peptide of the present invention can be administered to a subject to overcome tumor-specific toler-

ance in the subject. If desired, the tumor cell can be transfected to express a combination of peptides. For example, tumor cells obtained from a patient can be transfected *ex vivo* with an expression vector directing the expression of a peptide having B7-2-like activity alone, or in conjunction with a peptide having B7-1-like activity and/or B7-3-like activity. The transfected tumor cells are returned to the patient to result in expression of the peptides on the surface of the transfected cell. Alternatively, gene therapy techniques can be used to target a tumor cell for transfection *in vivo*.

The presence of the peptide of the present invention having the activity of a B lymphocyte antigen(s) on the surface of the tumor cell provides the necessary costimulation signal to T cells to induce a T cell mediated immune response against the transfected tumor cells. In addition, tumor cells which lack MHC class I or MHC class II molecules, or which fail to reexpress sufficient mounts of MHC class I or MHC class II molecules, can be transfected with nucleic acid encoding all or a portion of (e.g., a cytoplasmic-domain truncated portion) of an MHC class I α chain protein and β_2 microglobulin protein or an MHC class II α chain protein and an MHC class II β chain protein to thereby express MHC class I or MHC class II proteins on the cell surface. Expression of the appropriate class I or class II MHC in conjunction with a peptide having the activity of a B lymphocyte antigen (e.g., B7-1, B7-2, B7-3) induces a T cell mediated immune response against the transfected tumor cell. Optionally, a gene encoding an antisense construct which blocks expression of an MHC class II associated protein, such as the invariant chain, can also be cotransfected with a DNA encoding a peptide having the activity of a B lymphocyte antigen to promote presentation of tumor associated antigens and induce tumor specific immunity. Thus, the induction of a T cell mediated immune response in a human subject may be sufficient to overcome tumor-specific tolerance in the subject.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for thymocyte or splenocyte cytotoxicity include, without limitation, those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, *In Vitro* assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, *Immunologic studies in Humans*); Herrmann et al., *Proc. Natl. Acad. Sci. USA* 78:2488-2492, 1981; Herrmann et al., *J. Immunol.* 128:1968-1974, 1982; Handa et al., *J. Immunol.* 135:1564-1572, 1985; Takai et al., *J. Immunol.* 137:3494-3500, 1986; Takai et al., *J. Immunol.* 140:508-512, 1988; Herrmann et al., *Proc. Natl. Acad. Sci. USA* 78:2488-2492, 1981; Herrmann et al., *J. Immunol.* 128:1968-1974, 1982; Handa et al., *J. Immunol.* 135:1564-1572, 1985; Takai et al., *J. Immunol.* 137:3494-3500, 1986; Bowman et al., *J. Virology* 61:1992-1998; Takai et al., *J. Immunol.* 140:508-512, 1988; Bertagnoli et al., *Cellular Immunology* 133:327-341, 1991; Brown et al., *J. Immunol.* 153:3079-3092, 1994.

Assays for T-cell-dependent immunoglobulin responses and isotype switching (which will identify, among others, proteins that modulate T-cell dependent antibody responses and that affect Th1/Th2 profiles) include, without limitation, those described in: Maliszewski, *J. Immunol.* 144:3028-3033, 1990; and Assays for B cell function: *In vitro* antibody production, Mond, J. J. and Brunswick, M. In *Current Protocols in Immunology*, J. E. Coligan eds. Vol 1 pp. 3.8.1-3.8.16, John Wiley and Sons, Toronto. 1994.

Mixed lymphocyte reaction (MLR) assays (which will identify, among others, proteins that generate predominantly Th1 and CTL responses) include, without limitation, those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, *In Vitro* assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, *Immunologic studies in Humans*); Takai et al., *J. Immunol.* 137:3494-3500, 1986; Takai et al., *J. Immunol.* 140:508-512, 1988; Bertagnoli et al., *J. Immunol.* 149:3778-3783, 1992.

Dendritic cell-dependent assays (which will identify, among others, proteins expressed by dendritic cells that activate naive T-cells) include, without limitation, those described in: Guery et al., *J. Immunol.* 134:536-544, 1995; Inaba et al., *Journal of Experimental Medicine* 173:549-559, 1991; Macatonia et al., *Journal of Immunology* 154:5071-5079, 1995; Porgador et al., *Journal of Experimental Medicine* 182:255-260, 1995; Nair et al., *Journal of Virology* 67:4062-4069, 1993; Huang et al., *Science* 264:961-965, 1994; Macatonia et al., *Journal of Experimental Medicine* 169:1255-1264, 1989; Bhardwaj et al., *Journal of Clinical Investigation* 94:797-807, 1994; and Inaba et al., *Journal of Experimental Medicine* 172:631-640, 1990.

Assays for lymphocyte survival/apoptosis (which will identify, among others, proteins that prevent apoptosis after superantigen induction and proteins that regulate lymphocyte homeostasis) include, without limitation, those described in: Darzynkiewicz et al., *Cytometry* 13:795-808, 1992; Gorczyca et al., *Leukemia* 7:659-670, 1993; Gorczyca et al., *Cancer Research* 53:1945-1951, 1993; Itoh et al., *Cell* 66:233-243, 1991; Zacharchuk, *Journal of Immunology* 145:4037-4045, 1990; Zamai et al., *Cytometry* 14:891-897, 1993; Gorczyca et al., *International Journal of Oncology* 1:639-648, 1992.

Assays for proteins that influence early steps of T-cell commitment and development include, without limitation, those described in: Antica et al., *Blood* 84:111-117, 1994; Fine et al., *Cellular Immunology* 155:111-122, 1994; Galy et al., *Blood* 85:2770-2778, 1995; Toki et al., *Proc. Nat. Acad. Sci. USA* 88:7548-7551, 1991.

Hematopoiesis Regulating Activity

A protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis, e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility, for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to stimulate the production of erythroid precursors and/or erythroid cells; in supporting the growth and proliferation of myeloid cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelosuppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentary to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility in various stem cell disorders (such as

those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either in-vivo or ex-vivo (i.e., in conjunction with bone marrow transplantation or with peripheral progenitor cell transplantation (homologous or heterologous)) as normal cells or genetically manipulated for gene therapy.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

Assays for embryonic stem cell differentiation (which will identify, among others, proteins that influence embryonic differentiation hematopoiesis) include, without limitation, those described in: Johansson et al. *Cellular Biology* 15:141-151, 1995; Keller et al., *Molecular and Cellular Biology* 13:473-486, 1993; McClanahan et al., *Blood* 81:2903-2915, 1993.

Assays for stem cell survival and differentiation (which will identify, among others, proteins that regulate lymphohematopoiesis) include, without limitation, those described in: Methylcellulose colony forming assays, Freshney, M. G. In *Culture of Hematopoietic Cells*. R. I. Freshney, et al. eds. Vol pp. 265-268, Wiley-Liss, Inc., New York, N.Y. 1994; Hirayama et al., *Proc. Natl. Acad. Sci. USA* 89:5907-5911, 1992; Primitive hematopoietic colony forming cells with high proliferative potential, McNiece, I. K. and Briddell, R. A. In *Culture of Hematopoietic Cells*. R. I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, N.Y. 1994; Neben et al., *Experimental Hematology* 22:353-359, 1994; Cobblestone area forming cell assay, Ploemacher, R. E. In *Culture of Hematopoietic Cells*. R. I. Freshney, et al. eds. Vol pp. 1-21, Wiley-Liss, Inc., New York, N.Y. 1994; Long term bone marrow cultures in the presence of stromal cells, Spooner, E., Dexter, M. and Allen, T. In *Culture of Hematopoietic Cells*. R. I. Freshney, et al. eds. Vol pp. 163-179, Wiley-Liss, Inc., New York, N.Y. 1994; Long term culture initiating cell assay, Sutherland, H. J. In *Culture of Hematopoietic Cells*. R. I. Freshney, et al. eds. Vol pp. 139-162, Wiley-Liss, Inc., New York, N.Y. 1994.

Tissue Growth Activity

A protein of the present invention also may have utility in compositions used for bone, cartilage, tendon, ligament and/or nerve tissue growth or regeneration, as well as for wound healing and tissue repair and replacement, and in the treatment of burns, incisions and ulcers.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage damage or defects in humans and other animals. Such a preparation employing a protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery.

A protein of this invention may also be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A protein of the invention may also be useful in the treatment of osteoporosis or osteoarthritis, such as through stimulation of bone and/or cartilage repair or by blocking inflammation or processes of tissue destruction

(collagenase activity, osteoclast activity, etc.) mediated by inflammatory processes.

Another category of tissue regeneration activity that may be attributable to the protein of the present invention is tendon/ligament formation. A protein of the present invention, which induces tendon/ligament-like tissue or other tissue formation in circumstances where such tissue is not normally formed, has application in the healing of tendon or ligament tears, deformities and other tendon or ligament defects in humans and other animals. Such a preparation employing a tendon/ligament-like tissue inducing protein may have prophylactic use in preventing damage to tendon or ligament tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. De novo tendon/ligament-like tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other tendon or ligament defects of other origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments. The compositions of the present invention may provide environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon/ligament cells or progenitors ex vivo for return in vivo to effect tissue repair. The compositions of the invention may also be useful in the treatment of tendinitis, carpal tunnel syndrome and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

The protein of the present invention may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue, i.e. for the treatment of central and peripheral nervous system diseases and neuropathies, as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue. More specifically, a protein may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions which may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a protein of the invention.

Proteins of the invention may also be useful to promote better or faster closure of non-healing wounds, including without limitation pressure ulcers, ulcers associated with vascular insufficiency, surgical and traumatic wounds, and the like.

It is expected that a protein of the present invention may also exhibit activity for generation or regeneration of other tissues, such as organs (including, for example, pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac) and vascular (including vascular endothelium) tissue, or for promoting the growth of cells comprising such tissues. Part of the desired effects may be by inhibition or modulation of fibrotic scarring to allow normal tissue to regenerate. A protein of the invention may also exhibit angiogenic activity.

A protein of the present invention may also be useful for gut protection or regeneration and treatment of lung or liver fibrosis, reperfusion injury in various tissues, and conditions resulting from systemic cytokine damage.

A protein of the present invention may also be useful for promoting or inhibiting differentiation of tissues described above from precursor tissues or cells; or for inhibiting the growth of tissues described above.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for tissue generation activity include, without limitation, those described in: International Patent Publication No. WO95/16035 (bone, cartilage, tendon); International Patent Publication No. WO95/05846 (nerve, neuronal); International Patent Publication No. WO91/07491 (skin, endothelium).

Assays for wound healing activity include, without limitation, those described in: Winter, *Epidermal Wound Healing*, pps. 71-112 (Maibach, H. I. and Rovee, D. T., eds.), Year Book Medical Publishers, Inc., Chicago, as modified by Eaglstein and Mertz, J. Invest. Dermatol. 71:382-84 (1978).

Activin/Inhibin Activity

A protein of the present invention may also exhibit activin- or inhibin-related activities. Inhibins are characterized by their ability to inhibit the release of follicle stimulating hormone (FSH), while activins are characterized by their ability to stimulate the release of follicle stimulating hormone (FSH). Thus, a protein of the present invention, alone or in heterodimers with a member of the inhibin α family, may be useful as a contraceptive based on the ability of inhibins to decrease fertility in female mammals and decrease spermatogenesis in male mammals. Administration of sufficient amounts of other inhibins can induce infertility in these mammals. Alternatively, the protein of the invention, as a homodimer or as a heterodimer with other protein subunits of the inhibin- β group, may be useful as a fertility inducing therapeutic, based upon the ability of activin molecules in stimulating FSH release from cells of the anterior pituitary. See, for example, U.S. Pat. No. 4,798, 885. A protein of the invention may also be useful for advancement of the onset of fertility in sexually immature mammals, so as to increase the lifetime reproductive performance of domestic animals such as cows, sheep and pigs.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for activin/inhibin activity include, without limitation, those described in: Vale et al., *Endocrinology* 91:562-572, 1972; Ling et al., *Nature* 321:779-782, 1986; Vale et al., *Nature* 321:776-779, 1986; Mason et al., *Nature* 318:659-663, 1985; Forage et al., *Proc. Natl. Acad. Sci. USA* 83:3091-3095, 1986.

Chemotactic/Chemokinetic Activity

A protein of the present invention may have chemotactic or chemokinetic activity (e.g., act as a chemokine) for mammalian cells, including, for example, monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells. Chemotactic and chemokinetic proteins can be used to mobilize or attract a desired cell population to a desired site of action. Chemotactic or chemokinetic proteins provide particular advantages in treatment of wounds and other trauma to tissues, as well as in treatment of localized infections. For example, attraction of lymphocytes, monocytes or neutrophils to tumors or sites of infection may result in improved immune responses against the tumor or infecting agent.

A protein or peptide has chemotactic activity for a particular cell population if it can stimulate, directly or indirectly, the directed orientation or movement of such cell population. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a

particular protein has chemotactic activity for a population of cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for chemotactic activity (which will identify proteins that induce or prevent chemotaxis) consist of assays that measure the ability of a protein to induce the migration of cells across a membrane as well as the ability of a protein to induce the adhesion of one cell population to another cell population. Suitable assays for movement and adhesion include, without limitation, those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 6.12, Measurement of alpha and beta Chemokines 6.12.1-6.12.28; Taub et al. *J. Clin. Invest.* 95:1370-1376, 1995; Lind et al. *APMIS* 103:140-146, 1995; Muller et al. *Eur. J. Immunol.* 25:1744-1748; Gruber et al. *J. of Immunol.* 152:5860-5867, 1994; Johnston et al. *J. of Immunol.* 153:1762-1768, 1994.

Hemostatic and Thrombolytic Activity

A protein of the invention may also exhibit hemostatic or thrombolytic activity. As a result, such a protein is expected to be useful in treatment of various coagulation disorders (including hereditary disorders, such as hemophilias) or to enhance coagulation and other hemostatic events in treating wounds resulting from trauma, surgery or other causes. A protein of the invention may also be useful for dissolving or inhibiting formation of thromboses and for treatment and prevention of conditions resulting therefrom (such as, for example, infarction of cardiac and central nervous system vessels (e.g., stroke).

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assay for hemostatic and thrombolytic activity include, without limitation, those described in: Linet et al., *J. Clin. Pharmacol.* 26:131-140, 1986; Burdick et al., *Thrombosis Res.* 45:413-419, 1987; Humphrey et al., *Fibrinolysis* 5:71-79 (1991); Schaub, *Prostaglandins* 35:467-474, 1988.

Receptor/Ligand Activity

A protein of the present invention may also demonstrate activity as receptors, receptor ligands or inhibitors or agonists of receptor/ligand interactions. Examples of such receptors and ligands include, without limitation, cytokine receptors and their ligands, receptor kinases and their ligands, receptor phosphatases and their ligands, receptors involved in cell-cell interactions and their ligands (including without limitation, cellular adhesion molecules (such as selectins, integrins and their ligands) and receptor/ligand pairs involved in antigen presentation, antigen recognition and development of cellular and humoral immune responses). Receptors and ligands are also useful for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction. A protein of the present invention (including, without limitation, fragments of receptors and ligands) may themselves be useful as inhibitors of receptor/ligand interactions.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for receptor-ligand activity include without limitation those described in: *Current Protocols in Immunology*, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 7.28, Measurement of Cellular Adhesion under static conditions 7.28.1-7.28.22), Takai et al., *Proc. Natl. Acad. Sci. USA*

84:6864-6868, 1987; Bierer et al., J. Exp. Med. 168:1145-1156, 1988; Rosenstein et al., J. Exp. Med. 169:149-160 1989; Stoltenberg et al., J. Immunol. Methods 175:59-68, 1994; Stitt et al., Cell 80:661-670, 1995.

Anti-Inflammatory Activity

Proteins of the present invention may also exhibit anti-inflammatory activity. The anti-inflammatory activity may be achieved by providing a stimulus to cells involved in the inflammatory response, by inhibiting or promoting cell-cell interactions (such as, for example, cell adhesion), by inhibiting or promoting chemotaxis of cells involved in the inflammatory process, inhibiting or promoting cell extravasation, or by stimulating or suppressing production of other factors which more directly inhibit or promote an inflammatory response. Proteins exhibiting such activities can be used to treat inflammatory conditions including chronic or acute conditions), including without limitation intimation associated with infection (such as septic shock, sepsis or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine-induced lung injury, inflammatory bowel disease, Crohn's disease or resulting from over production of cytokines such as TNF or IL-1. Proteins of the invention may also be useful to treat anaphylaxis and hypersensitivity to an antigenic substance or material.

Tumor Inhibition Activity

In addition to the activities described above for immunological treatment or prevention of tumors, a protein of the invention may exhibit other anti-tumor activities. A protein may inhibit tumor growth directly or indirectly (such as, for example, via ADCC). A protein may exhibit its tumor inhibitory activity by acting on tumor tissue or tumor precursor tissue, by inhibiting formation of tissues necessary to support tumor growth (such as, for example, by inhibiting angiogenesis), by causing production of other factors, agents or cell types which inhibit tumor growth, or by suppressing, eliminating or inhibiting factors, agents or cell types which promote tumor growth.

Other Activities

A protein of the invention may also exhibit one or more of the following additional activities or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, without limitation, bacteria, viruses, fungi and other parasites; effecting (suppressing or enhancing) bodily characteristics, including, without limitation, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or body part size or shape (such as, for example, breast augmentation or diminution, change in bone form or shape); effecting biorhythms or circadian cycles or rhythms; effecting the fertility of male or female subjects; effecting the metabolism, catabolism, anabolism, processing, utilization, storage or elimination of dietary fat, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional factors or component(s); effecting behavioral characteristics, including, without limitation, appetite, libido, stress, cognition (including cognitive disorders), depression (including depressive disorders) and violent behaviors; providing analgesic effects or other pain reducing effects; promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages; hormonal or endocrine activity; in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases; treatment of hyperproliferative disorders (such as, for example, psoriasis); immunoglobulin-like activity (such as, for example, the ability to bind antigens or complement); and the ability to act

as an antigen in a vaccine composition to raise an immune response against such protein or another material or entity which is cross-reactive with such protein.

ADMINISTRATION AND DOSING

A protein of the present invention (from whatever source derived, including without limitation from recombinant and non-recombinant sources) may be used in a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may also contain (in addition to protein and a carrier) diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, TNF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IFN, TNF α , TNF β , G-CSF, Meg-CSF, thrombopoietin, stem cell factor, and erythropoietin. The pharmaceutical composition may further contain other agents which either enhance the activity of the protein or complement its activity or use in treatment. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with protein of the invention, or to minimize side effects. Conversely, protein of the present invention may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

A protein of the present invention may be active in multimers (e.g., heterodimers or homodimers) or complexes with itself or other proteins. As a result, pharmaceutical compositions of the invention may comprise a protein of the invention in such multimeric or complexed form.

The pharmaceutical composition of the invention may be in the form of a complex of the protein(s) of present invention along with protein or peptide antigens. The protein and/or peptide antigen will deliver a stimulatory signal to both B and T lymphocytes. B lymphocytes will respond to antigen through their surface immunoglobulin receptor. T lymphocytes will respond to antigen through the T cell receptor (TCR) following presentation of the antigen by MHC proteins. MHC and structurally related proteins including those encoded by class I and class II MHC genes on host cells will serve to present the peptide antigen(s) to T lymphocytes. The antigen components could also be supplied as purified MHC-peptide complexes alone or with co-stimulatory molecules that can directly signal T cells. Alternatively antibodies able to bind surface immunoglobulin and other molecules on B cells as well as antibodies able to bind the TCR and other molecules on T cells can be combined with the pharmaceutical composition of the invention.

The pharmaceutical composition of the invention may be in the form of a liposome in which protein of the present invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers in aqueous solution. Suitable lipids for liposomal formulation include,

without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, i.e., treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of protein of the present invention is administered to a mammal having a condition to be treated. Protein of the present invention may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, protein of the present invention may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering protein of the present invention in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of protein of the present invention used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, topical application or cutaneous, subcutaneous, intraperitoneal, parenteral or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of protein of the present invention is administered orally, protein of the present invention will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% protein of the present invention, and preferably from about 25 to 90% protein of the present invention. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of protein of the present invention, and preferably from about 1 to 50% protein of the present invention.

When a therapeutically effective amount of protein of the present invention is administered by intravenous, cutaneous

or subcutaneous injection, protein of the present invention will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to protein of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of protein of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of protein of the present invention with which to treat each individual patient. Initially, the attending physician will administer low doses of protein of the present invention and observe the patient's response. Larger doses of protein of the present invention may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.01 µg to about 100 mg (preferably about 0.1 µg to about 10 mg, more preferably about 0.1 µg to about 1 mg) of protein of the present invention per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the protein of the present invention will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the protein. Such antibodies may be obtained using either the entire protein or fragments thereof as an immunogen. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Methods for synthesizing such peptides are known in the art, for example, as in R. P. Merrifield, *J. Amer. Chem. Soc.* 85, 2149-2154 (1963); J. L. Krstenansky, et al., *FEBS Lett.* 211, 10 (1987). Monoclonal antibodies binding to the protein of the invention may be useful diagnostic agents for the immunodetection of the protein. Neutralizing monoclonal antibodies binding to the protein may also be useful therapeutics for both conditions associated with the protein and also in the treatment of some forms of cancer where abnormal expression of the protein is involved. In the case of cancerous cells or leukemic cells, neutralizing monoclonal antibodies against the protein may be useful in detecting and preventing the metastatic spread of the cancerous cells, which may be mediated by the protein.

For compositions of the present invention which are useful for bone, cartilage, tendon or ligament regeneration,

the therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than a protein of the invention which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering the protein-containing composition to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyglycolic acid and polyanhydrides. Other potential materials are biodegradable and biologically well-defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

Presently preferred is a 50:50 (mole weight) copolymer of lactic acid and glycolic acid in the form of porous particles having diameters ranging from 150 to 800 microns. In some applications, it will be useful to utilize a sequestering agent, such as carboxymethyl cellulose or autologous blood clot, to prevent the protein compositions from disassociating from the matrix.

A preferred family of sequestering agents is cellulosic materials such as alkylcelluloses (including hydroxyalkylcelluloses), including methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, and carboxymethylcellulose, the most preferred being cationic salts of carboxymethylcellulose (CMC). Other pre-

ferred sequestering agents include hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer and poly(vinyl alcohol). The amount of sequestering agent useful herein is 0.5-20 wt %, preferably 1-10 wt % based on total formulation weight, which represents the amount necessary to prevent desorption of the protein from the polymer matrix and to provide appropriate handling of the composition, yet not so much that the progenitor cells are prevented from infiltrating the matrix, thereby providing the protein the opportunity to assist the osteogenic activity of the progenitor cells.

In further compositions, proteins of the invention may be combined with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF- α and TGF- β), and insulin-like growth factor (IGF).

The therapeutic compositions are also presently valuable for veterinary applications. Particularly domestic animals and thoroughbred horses, in addition to humans, are desired patients for such treatment with proteins of the present invention.

The dosage regimen of a protein-containing pharmaceutical composition to be used in tissue regeneration will be determined by the attending physician considering various factors which modify the action of the proteins, e.g., amount of tissue weight desired to be formed, the site of damage, the condition of the damaged tissue, the size of a wound, type of damaged tissue (e.g., bone), the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and with inclusion of other proteins in the pharmaceutical composition. For example, the addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage. Progress can be monitored by periodic assessment of tissue/bone growth and/or repair, for example, X-rays, histomorphometric determinations and tetracycline labeling.

Polynucleotides of the present invention can also be used for gene therapy. Such polynucleotides can be introduced either in vivo or ex vivo into cells for expression in a mammalian subject. Polynucleotides of the invention may also be administered by other known methods for introduction of nucleic acid into a cell or organism (including, without limitation, in the form of viral vectors or naked DNA).

Cells may also be cultured ex vivo in the presence of proteins of the present invention in order to proliferate or to produce a desired effect on or activity in such cells. Treated cells can then be introduced in vivo for therapeutic purposes.

Patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 11

(2) INFORMATION FOR SEQ ID NO:1:

-continued

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 432 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

GGTTTGAAAA CTCTGCTTCC TTTGTGAATT TGGTGTTAGG AGTTCTTATT GTTATTCTGC      60
AGCCTTTACT ATTGTCCTTT ATTTACTGAA CACAGTGAAT ACCAAGCACT GTTTATTAGA      120
GGTTAAGGAGT AGGGGCAAGT GATTAAAAAA ACAAAAAAGC TAATAATCTC CTCAAGCAAT      180
TTCTGOCCTA ATAGAATTAT AGTAGACAGT GAAGTATCTA AACCCAGGGA ATCAGATTGA      240
GGCACCATGT CCATCGCCTT GAGAATTAAT AGGCTGCATT TCTGGGTTCT CCNTTTTTTT      300
TTTTTTTTTG CCCAACTGAG TCTTCTGTG GACTTACATG GAACTTCTTA TTCTCTTAAA      360
TCATTAAGTT ACTTGACAAT ATTCTTGGAT TTGGAGAAAC TGGATGTAGG GCCGTATGAA      420
AAAAATCATT GA                                     432

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ser Ile Ala Leu Arg Ile Asn Arg Leu His Phe Trp Val Leu Xaa      15
1      5      10
Phe Phe Phe Phe Phe Ala Gln Leu Ser Leu Ser Val Asp Leu His Gly      30
20      25      30
Thr Ser Tyr Ser Leu Lys Ser Leu Ser Tyr Leu Thr Ile Phe Leu Asp      45
35      40      45
Leu Glu Lys Leu Asp Val Gly Pro Tyr Glu Lys Ile Ile Arg      60
50      55      60

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 219 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

ATAGGATACN GTATCTNGCT TTTTCATTT AAACGTCGNG AGCAATTTTC CCAAGACATA      60
ACAAACTGTC TTGAAAAAAN GGAAAAACATT NGGGGCTGTC AGCANAACNG AAAATGTTTT      120
CTGGGTGAGA CACATGTATC TTGNAATGG GTTGGATTTA GTGTGCTTTA TTTCAATAAA      180
AATTCAGTAT TATAATTTAA AAAAAAAAAA AAAAAAAAAA      219

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 501 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

TCCACAGGTG TCCANTCCCA GGTCCAAC TG CAGATTTCGA ATTCGGCCTT CATGGCCTAG 60
AGCGACGCGG AGAARAAGCTC CGGGTGCCGC GGCAC TG CAG CGCTGAGATT CCTTTACAAA 120
GAAACTCAGA GGACCGGGAA GAAAGAATTT CACCTTTGCG ACGTGCTAGA AAATAARGTC 180
GTCTGGGAAA AGGACTGGAG ACACAAGCGC ATCSCAAS Y Y SRGTGAAGGA SAAASNGAKG 240
GANBTAKWWM MGWGSWGA AAAATKT Y WWKC AAMMWMGGTA TTTTCCCTTG GATATTAAC 300
TGCATATCTG AAGAAATGGC ATTCCGACACA ATTTGCGTGT TGGTTGGAAT ATTTATTTGT 360
TCTATCTGTG TGAAGGATC TTCCCAAGCCC CAAAGCAAGAG TTTATTTAAC ATTTGATGAA 420
CTTCGAGAAA CCAAGACCTC TGAATACTTC AGCCTTTCCC ACCATCCTTT AGACTACAGG 480
ATTTTATTAA TGGATGAAGA T 501

```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ala Phe Arg Thr Ile Cys Val Leu Val Gly Val Phe Ile Cys Ser
1      5      10      15
Ile Cys Val Lys Gly Ser Ser Gln Pro Gln Ala Arg Val Tyr Leu Thr
20     25     30
Phe Asp Glu Leu Arg Glu Thr Lys Thr Ser Glu Tyr Phe Ser Leu Ser
35     40     45
His His Pro Leu Asp Tyr Arg Ile Leu Leu Met Asp Glu Asp
50     55     60

```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 302 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

CTAOCAC TAG ACATGTCATG GTCTTCATGG TGCATATAAA TATATTTAAC TTAACCCAGA 60
TTTTATTTAT ATCTTTATTC ACCTTTTCTT CAAAATCGAT ATGGTGGCTG CAAAAC TAGA 120
ATTGTTGCAT CCTCAATNG AATGAGGGCC ATATCCCTGT GGTATTCCTT TCCTGCTTNG 180
GGGCTTTAGA ATTCTAATG TCAGTGATTT TGTATATGAA AACAA GTTCC AAATCCACAG 240
CTTTTACGTA GTAAAAGTCA TAAATGCATA TGACAGAATG GCTATCAAAA GAAAAAAAAA 300
AA 302

```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 448 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

GGCGAARGCA  GCGGCAGGTC  GGGAGCAARA  TGGCCTGCG  GCCAGGAGCT  GGTTCCTGGT  60
GCGGCGGGGC  CCGGARGAKY  ATR-
RYGYGRK  KTYRYYSKG  KEWKSMGGST  TCATGTTTCC  120
TGTTGCAGGT  GGGATAAGAC  CCCCTCAAGG  CCTGATGCCG  ATGCAGCAAC  AAGGATTTC  180
TATGGTCTCT  GTCATGCAGC  CTAATATGCA  AGGCATTATG  GGAATGAATT  ACAGCTCTCA  240
GATGTCCCAA  GGACCTATTG  CTATGCAGGC  AGGAATACCA  ATGGGACCAA  TGCCAGCAGC  300
GGGAATGCCT  TACCTAGGAC  AAGCACCCCT  CCTGGGCATG  CGTCCTCCAG  GCCCACAGTA  360
CACTCCAGAC  ATGCAGAAGC  AGTTTGCCGA  AGAGCAGCAG  AAACGATTTG  AACAGCAGCA  420
AAAACTCTTA  GAAAAAAAAA  AAAAAAAAAA  448

```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met  Phe  Pro  Val  Ala  Gly  Gly  Ile  Arg  Pro  Pro  Gln  Gly  Leu  Met  Pro
1          5          10          15
Met  Gln  Gln  Gln  Gly  Phe  Pro  Met  Val  Ser  Val  Met  Gln  Pro  Asn  Met
20          25          30
Gln  Gly  Ile  Met  Gly  Met  Asn  Tyr  Ser  Ser  Gln  Met  Ser  Gln  Gly  Pro
35          40          45
Ile  Ala  Met  Gln  Ala  Gly  Ile  Pro  Met  Gly  Pro  Met  Pro  Ala  Ala  Gly
50          55          60
Met  Pro  Tyr  Leu  Gly  Gln  Ala  Pro  Phe  Leu  Gly  Met  Arg  Pro  Pro  Gly
65          70          75          80
Pro  Gln  Tyr  Thr  Pro  Asp  Met  Gln  Lys  Gln  Phe  Ala  Glu  Glu  Gln  Gln
85          90          95
Lys  Arg  Phe  Glu  Gln  Gln  Gln  Lys  Leu  Leu  Glu
100          105

```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GNGCCTCAAT CTGATTCCCT GGGTTTAGA

29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ONCCGGAATG CCATTTCTTC AGATATGCA

29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TNCCATTGGT ATTCTGCCT GCATAGCAA

29

What is claimed is:

1. An isolated polynucleotide selected from the group consisting of:
 - (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1;
 - (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 247 to nucleotide 432;
 - (c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 328 to nucleotide 432;
 - (d) a polynucleotide comprising the nucleotide sequence of the full length protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146;
 - (e) a polynucleotide encoding the full length protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146;
 - (f) a polynucleotide comprising the nucleotide sequence of the mature protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146;
 - (g) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146; and
 - (h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:2.
2. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1.
3. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 247 to nucleotide 432.
4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 328 to nucleotide 432.
5. The polynucleotide of claim 1 comprising the nucleotide sequence of the full length protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146.
6. The polynucleotide of claim 1 encoding the full length protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146.
7. The polynucleotide of claim 1 comprising the nucleotide sequence of the mature protein coding sequence of clone BD372_5 deposited under accession number ATCC 98146.
8. The polynucleotide of claim 1 encoding the mature protein encoded by the cDNA insert of clone BD372_5 deposited under accession number ATCC 98146.
9. The polynucleotide of claim 1 encoding a protein comprising the amino acid sequence of SEQ ID NO:2.
10. A vector comprising a polynucleotide of claim 1 wherein said polynucleotide is operably linked to an expression control sequence.
11. A host cell transformed with a vector of claim 2.
12. The host cell of claim 3, wherein said cell is a mammalian cell.
13. A process for producing a protein, which comprises:
 - (a) growing a culture of the host cell of claim 3 in a suitable culture medium; and
 - (b) purifying the protein from the culture.
14. An isolated gene corresponding to the cDNA sequence of SEQ ID NO:1.

* * * * *

N

This Page Blank (uspto)



US005552281A

United States Patent [19]

Stashenko et al.

[11] Patent Number: 5,552,281

[45] Date of Patent: Sep. 3, 1996

[54] HUMAN OSTEOCLAST-SPECIFIC AND -RELATED GENES

[75] Inventors: Philip Stashenko, Norfolk; Yi-Ping Li, Boston; Anne L. Wucherpfennig, Brookline, all of Mass.

[73] Assignee: Forsyth Dental Infirmary for Children, Boston, Mass.

[21] Appl. No.: 392,678

[22] Filed: Feb. 23, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 45,270, Apr. 6, 1993, abandoned.

[51] Int. Cl.⁶ C07H 21/04; C12N 5/10; C12N 15/70; C12Q 1/68

[52] U.S. Cl. 435/6; 435/69.1; 435/172.3; 435/252.3; 435/320.1; 536/23.1

[58] Field of Search 435/6, 320.1, 252.3, 435/69.1, 172.3; 536/23.1

[56] References Cited

PUBLICATIONS

Blair, Harry C., et al., "Extracellular-matrix degradation at acid pH. Avian osteoclast acid collagenase isolation and characterization", *Biochemical Journal* 290(3):873-884 (15 Mar. 1993).

Tezuka, Ken-Ichi, et al., "Identification of osteopontin in isolated rabbit osteoclasts", *Biochemical and Biophysical Research Communications* 186(2):914-916 (31 Jul. 1992).

Tezuka, Ken-Ichi, et al., "Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts", *Journal of Biological Chemistry* 269(2):1106-1108, (14 Jan. 1994).

Horton, Michael A. et al., "Monoclonal Antibodies to Osteoclastomas (Giant Cell Bone Tumors): Definition of Osteoclast-specific Cellular Antigens," *Cancer Research* 45, 5663-5669 (Nov. 1985).

Davies, John et al., "The Osteoclast Functional Antigen, Implicated in the Regulation of Bone Resorption, Is Biochemically Related to the Vitronectin Receptor," *The Journal of Cell Biology* 109, 1817-1826 (Oct. 1989).

Hayman, Alison, R. et al., "Purification and characterization of a tartrate-resistant acid phosphatase from human osteoclastomas," *Biochem. J.* 261, 601-609 (1989).

Sandberg, M. et al., "Localization of the Expression of Types I, III, and IV Collagen, TGF- β 1 and c-fos Genes in Developing Human Calvarial Bones," *Developmental Biology* 130, 324-334 (1988).

Sandberg, M. et al., "Enhanced expression of TGF- β and c-fos mRNAs in the growth plates of developing human long bones," *Development* 102, 461-470 (1988).

Ek-Rylander, Barbro et al., "Cloning, Sequence, and Developmental Expression of a Type 5, Tartrate-resistant, Acid Phosphatase of Rat Bone," *The Journal of Biological Chemistry* 266(36), 24684-24689 (Dec. 25, 1991).

GenBank/EMBL Sequence Search Printout, pp. 1-19 (Jun. 24, 1993).

Primary Examiner—W. Gary Jones

Assistant Examiner—Paul B. Tran

Attorney, Agent, or Firm—Hamilton, Brook, Smith & Reynolds, P.C.

[57]

ABSTRACT

The present invention relates to purified DNA sequences encoding all or a portion of an osteoclast-specific or -related gene products and a method for identifying such sequences. The invention also relates to antibodies directed against an osteoclast-specific or -related gene product. Also claimed are DNA constructs capable of replicating DNA encoding all or a portion of an osteoclast-specific or -related gene product, and DNA constructs capable of directing expression in a host cell of an osteoclast-specific or -related gene product.

5 Claims, 1 Drawing Sheet

1 AACATCTT GCGTCTGG TGGCTCTT GCGTCTCT GCTTCTCTT TCTTCTCT
41 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
81 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
1961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
2961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
3961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
4961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
5961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
6961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
7961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
8961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9041 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9081 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9121 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9161 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9201 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9241 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9281 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9321 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9361 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9401 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9441 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9481 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9521 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9561 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9601 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9641 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9681 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9721 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9761 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9801 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9841 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9881 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9921 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
9961 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT
10001 GCGTCTCTT TCTTCTCTT GCGTCTCT GCGTCTCT GCTTCTCTT TCTTCTCT

1 AGACACCTCT GCCCTACCA TGAGCCTCTG GCAGCCCTG GTCCTGGTGC TCCTGGTGCT
61 GGGCTGCTGC TTTGCTGCCC CCAGACAGCG CCAGTCCACC CTTGTGCTCT TCCCTGGAGA
121 CCTGAGAACC AATCTCACC ACAGGCAGCT GGCAGAGGAA TACCTGTACC GCTATGGTTA
181 CACTCGGGTG GCAGAGATGC GTGGAGAGTC GAAATCTCTG GGGCCTGCGC TGCTGCTTCT
241 CCAGAAGCAA CTGTCCCTGC CCAGAGCCGG TGAGCTGGAT AGCGCCACGC TGAAGGCCAT
301 GCGAACCCCA CGGTGCGGGG TCCCAGACCT GGGCAGATT CAAACCTTTG AGGGCGACCT
361 CAAGTGGCAC CACCACAACA TCACCTATTG GATCCAAAC TACTCGGAAG ACTTGCCGCG
421 GCGCGTGATT GACGACGCCT TTGCCGCGC CTTGCACTG TGGAGCGCGG TGACGCCGCT
481 CACCTTCACT CGCGTGACA GCCGGGACGC AGACATCGTC ATCCAGTTTG GTGTCGCGGA
541 GCACGGAGAC GGGTATCCCT TCGACGGGAA GGACGGGCTC CTGGCACACG CTTTCTCTCC
601 TGGCCCCGGC ATTCAGGGAG ACGCCATT TCGACGATGAC GAGTTGTGGT CCCTGGGCAA
661 GGGCGTCGTG GTTCCAATC GGTTTGGAAA CGCAGATGGC GCGGCCGTGC ACTTCCCTT
721 CATCTTCGAG GGCCGCTCCT ACTCTGCCTG CACCACCGAC GGTGCTCCG ACGGGTTGCC
781 CTGGTGCACT ACCACGGCCA ACTACGACAC CGACGACGG TTTGGCTTCT GCCCCAGCGA
841 GAGACTCTAC ACCCGGGACG GCAATGCTGA TGGGAAACCC TGCCAGTTT CATTCTCTT
901 CCAAGGCCAA TCCTACTCCG CCTGCACCAC GGACGGTCG TCCGACGGCT ACCGCTGGTG
961 CGCCACCACC GCCAACTACG ACCGGGACAA GCTCTTCGGC TTCTGCCCGA CCCGAGCTGA
1021 CTGACGCGTG ATGGGGGGCA ACTCGGCGGG GGAGCTGTGC GTCTTCCCCT TCACCTTCCT
1081 GGGTAAGGAG TACTCGACCT GTACCAGCGA GGGCCGCGGA GATGGGCGCC TCTGGTGCGC
1141 TACCACCTCG AACTTTGACA GCGACAAGAA GTGGGGCTTC TGCCCGGACC AAGGATACAG
1201 TTTGTTCTC GTGGCGGCGC ATGAGTTCGG CCACGCGCTG GGCTTAGATC ATTCCTCAGT
1261 GCCGGAGGCG CTGATGTACC CTATGTACCG CTTCACTGAG GGGCCCCCT TGCATAAGGA
1321 CGACGTGAAT GGATCCGGC ACCTCTATGG TCCTCGCCCT GAACCTGAGC CACGGCCTCC
1381 AACCACCACC ACACCGCAGC CCACGGCTCC CCGACGGTC TGCCCCACCG GACCCCCAC
1441 TGTCCACCCC TCAGAGCGCC CCACAGTGG CCCACAGGT CCCCCCTCAG CTGGCCCCAC
1501 AGGTCCCCC ACTGCTGGCC CTTCTACGGC CACTACTGTG CCTTTGAGTC CGGTGGACGA
1561 TGCCTGCAAC GTGAACATCT TCGACGCCAT CGCGGAGATT GGAACCAGC TGTATTTGTT
1621 CAAGGATGGG AAGTACTGGC GATTCTCTGA GGCAGGGGG AGCCGGCCGC AGGGCCCCTT
1681 CTTTATCGCC GACAAGTGGC CCGCGCTGCC CCGCAAGCTG GACTCGGTCT TTGAGGAGCC
1741 GCTCTCCAAG AAGCTTTTCT TCTTCTCTGG GCGCCAGGTG TGGGTGTACA CAGGCGCGTC
1801 GGTGCTGGGC CCGAGGCGTC TGGACAAGCT GGGCCTGGGA GCGACGTGG CCCAGGTGAC
1861 CGGGGCCCTC CGGAGTGGCA GGGGGAAGAT GTGCTGTTT AGCGGGCGGC GCCTCTGGAG
1921 GTTCGACGTG AAGGCGCAGA TGGTGATCC CCGAGCGCC AGCGAGGTGG ACCGGATGTT
1981 CCCCAGGGTG CTTTGGACA CGCAGACGT CTTCCAGTAC CGAGAGAAAG CCTATTTCTG
2041 CCAGGACCGC TTCTACTGGC GCGTGAGTTC CCGGAGTGAG TTGAACCAGG TGGACCAAGT
2101 GGGCTACGTG ACCTATGACA TCCTGCACTG CCTGAGGAC TAGGGCTCCC GTCTGCTTT
2161 GCAGTGCCAT GTAAATCCCC ACTGGGACCA ACCCTGGGGA AGGAGCCAGT TTGCCGATA
2221 CAACTGGTA TTCTGTTCTG GAGGAAGGG AGGAGTGGAG GTGGGCTGG CCCTCTCTC
2281 TCACCTTTGT TTTTGTGG AGTGTCTTA ATAACTGG ATTCTTAAC CTTT

Figure 1

HUMAN OSTEOCLAST-SPECIFIC AND -RELATED GENES

RELATED APPLICATION

This application is a continuation of application Ser. No. 08/045,270 filed on Apr. 6, 1993 now abandoned.

BACKGROUND OF THE INVENTION

Excessive bone resorption by osteoclasts contributes to the pathology of many human diseases including arthritis, osteoporosis, periodontitis, and hypercalcemia of malignancy. During resorption, osteoclasts remove both the mineral and organic components of bone (Blair, H. C., et al., *J. Cell Biol.* 102:1164 (1986)). The mineral phase is solubilized by acidification of the sub-osteoclastic lacuna, thus allowing dissolution of hydroxyapatite (Vaes, G., *Clin. Orthop. Relat.* 231:239 (1988)). However, the mechanism(s) by which type I collagen, the major structural protein of bone, is degraded remains controversial. In addition, the regulation of osteoclastic activity is only partly understood. The lack of information concerning osteoclast function is due in part to the fact that these cells are extremely difficult to isolate as pure populations in large numbers. Furthermore, there are no osteoclastic cell lines available. An approach to studying osteoclast function that permits the identification of heretofore unknown osteoclast-specific or -related genes and gene products would allow identification of genes and gene products that are involved in the resorption of bone and in the regulation of osteoclastic activity. Therefore, identification of osteoclast-specific or -related genes or gene products would prove useful in developing therapeutic strategies for the treatment of disorders involving aberrant bone resorption.

SUMMARY OF THE INVENTION

The present invention relates to isolated DNA sequences encoding all or a portion of osteoclast-specific or -related gene products. The present invention further relates to DNA constructs capable of replicating DNA encoding osteoclast-specific or -related gene products. In another embodiment, the invention relates to a DNA construct capable of directing expression of all or a portion of the osteoclast-specific or -related gene product in a host cell.

Also encompassed by the present invention are prokaryotic or eukaryotic cells transformed or transfected with a DNA construct encoding all or a portion of an osteoclast-specific or -related gene product. According to a particular embodiment, these cells are capable of replicating the DNA construct comprising the DNA encoding the osteoclast-specific or -related gene product, and, optionally, are capable of expressing the osteoclast-specific or -related gene product. Also claimed are antibodies raised against osteoclast-specific or -related gene products, or portions of these gene products.

The present invention further embraces a method of identifying osteoclast-specific or -related DNA sequences and DNA sequences identified in this manner. In one embodiment, cDNA encoding osteoclast is identified as follows: First, human giant cell tumor of the bone was used to 1) construct a cDNA library; 2) produce ³²P-labelled cDNA to use as a stromal cell⁺; osteoclast⁻ probe, and 3) produce (by culturing) a stromal cell population lacking osteoclasts. The presence of osteoclasts in the giant cell tumor was confirmed by histological staining for the osteo-

clast marker, type 5 tartrate-resistant acid phosphatase (TRAP) and with the use of monoclonal antibody reagents.

The stromal cell population lacking osteoclasts was produced by dissociating cells of a giant cell tumor, then growing and passaging the cells in tissue culture until the cell population was homogeneous and appeared fibroblastic. The cultured stromal cell population did not contain osteoclasts. The cultured stromal cells were then used to produce a stromal cell⁺, osteoclast⁻ ³²P-labelled cDNA probe.

The cDNA library produced from the giant cell tumor of the bone was then screened in duplicate for hybridization to the cDNA probes: one screen was performed with the giant cell tumor cDNA probe (stromal cell⁺, osteoclast⁺), while a duplicate screen was performed using the cultured stromal cell cDNA probe (stromal cell⁺, osteoclast⁻). Hybridization to a stromal⁺, osteoclast⁺ probe, accompanied by failure to hybridize to a stromal⁺, osteoclast⁻ probe indicated that a clone contained nucleic acid sequences specifically expressed by osteoclasts.

In another embodiment, genomic DNA encoding osteoclast-specific or -related gene products is identified through known hybridization techniques or amplification techniques. In one embodiment, the present invention relates to a method of identifying DNA encoding an osteoclast-specific or -related protein, or gene product, by screening a cDNA library or a genomic DNA library with a DNA probe comprising one or more sequences selected from the group consisting of the DNA sequences set out in Table I (SEQ ID NOs: 1-32). Finally, the present invention relates to an osteoclast-specific or related protein encoded by a nucleotide sequence comprising a DNA sequence selected from the group consisting of the sequences set out in Table I, or their complementary strands.

BRIEF DESCRIPTION OF FIG. 1

The FIG. 1 shows cDNA sequence (SEQ ID NO: 33) of human gelatinase B, and highlights those portions of the sequence represented by the osteoclast-specific or -related cDNA clones of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

As described herein, Applicant has identified osteoclast-specific or osteoclast-related nucleic acid sequences. These sequences were identified as follows: Human giant cell tumor of the bone was used to 1) construct a cDNA library; 2) produce ³²P-labelled cDNA to use as a stromal cell⁺, osteoclast⁺ probe, and 3) produce (by culturing) a stromal cell population lacking osteoclasts. The presence of osteoclasts in the giant cell tumor was confirmed by histological staining for the osteoclast marker, type 5 acid phosphatase (TRAP). In addition, monoclonal antibody reagents were used to characterize the multinucleated cells in the giant cell tumor, which cells were found to have a phenotype distinct from macrophages and consistent with osteoclasts.

The stromal cell population lacking osteoclasts was produced by dissociating cells of a giant cell tumor, then growing the cells in tissue culture for at least five passages. After five passages the cultured cell population was homogeneous and appeared fibroblastic. The cultured population contained no multinucleated cells at this point, tested negative for type 5 acid phosphatase, and tested variably alkaline phosphatase positive. That is, the cultured stromal cell population did not contain osteoclasts. The cultured stromal

cells were then used to produce a stromal cell⁺, osteoclast⁻ ³²P-labelled cDNA probe.

The cDNA library produced from the giant cell tumor of the bone was then screened in duplicate for hybridization to the cDNA probes: one screen was performed with the giant cell tumor cDNA probe (stromal cell⁺, osteoclast⁻), while a duplicate screen was performed using the cultured stromal cell cDNA probe (stromal cell⁺ osteoclast⁻). Clones that hybridized to the giant cell tumor cDNA probe (stromal⁺, osteoclast⁻), but not to the stromal cell cDNA probe (stromal⁺, osteoclast⁻), were assumed to contain nucleic acid sequences specifically expressed by osteoclasts.

As a result of the differential screen described herein, DNA specifically expressed in osteoclast cells characterized as described herein was identified. This DNA, and equivalent DNA sequences, is referred to herein as osteoclast-specific or osteoclast-related DNA. Osteoclast-specific or -related DNA of the present invention can be obtained from sources in which it occurs in nature, can be produced recombinantly or synthesized chemically; it can be cDNA, genomic DNA, recombinantly-produced DNA or chemically-produced DNA. An equivalent DNA sequence is one which hybridizes, under standard hybridization conditions, to an osteoclast-specific or -related DNA identified as described herein or to a complement thereof.

Differential screening of a human osteoclastoma cDNA library was performed to identify genes specifically expressed in osteoclasts. Of 12,000 clones screened, 195 clones were identified which are either uniquely expressed in osteoclasts, or are osteoclast-related. These clones were further identified as osteoclast-specific, as evidenced by failure to hybridize to mRNA derived from a variety of unrelated human cell types, including epithelium, fibroblasts, lymphocytes, myelomonocytic cells, osteoblasts, and neuroblastoma cells. Of these, 32 clones contain novel cDNA sequences which were not found in the GenBank database.

A large number of cDNA clones obtained by this procedure were found to represent 92 kDa type IV collagenase (gelatinase B; E.C. 3.4.24.35) as well as tartrate resistant acid phosphatase. In situ hybridization localized mRNA for gelatinase B to multinucleated giant cells in human osteoclastomas. Gelatinase B immunoreactivity was demonstrated in giant cells from 8/8 osteoclastomas, osteoclasts in normal bone, and in osteoclasts of Paget's disease by use of a polyclonal antisera raised against a synthetic gelatinase B peptide. In contrast, no immunoreactivity for 72 kDa type IV collagenase (gelatinase A; E.C. 3.4.24.24), which is the product of a separate gene, was detected in osteoclastomas or normal osteoclasts.

The present invention has utility for the production and identification of nucleic acid probes useful for identifying osteoclast-specific or -related DNA. Osteoclast-specific or -related DNA of the present invention can be used to produce osteoclast-specific or -related gene products useful in the therapeutic treatment of disorders involving aberrant bone resorption. The osteoclast-specific or -related sequences are also useful for generating peptides which can then be used to produce antibodies useful for identifying osteoclast-specific or -related gene products, or for altering the activity of osteoclast-specific or -related gene products. Such antibodies are referred to as osteoclast-specific antibodies. Osteoclast-specific antibodies are also useful for identifying osteoclasts. Finally, osteoclast-specific or -related DNA sequences of the present invention are useful in gene therapy. For example, they can be used to alter the

expression in osteoclasts of an aberrant osteoclast-specific or -related gene product or to correct aberrant expression of an osteoclast-specific or -related gene product. The sequences described herein can further be used to cause osteoclast-specific or related gene expression in cells in which such expression does not ordinarily occur, i.e., in cells which are not osteoclasts.

Example 1—Osteoclast cDNA Library Construction

Messenger RNA (mRNA) obtained from a human osteoclastoma ('giant cell tumor of bone'), was used to construct an osteoclastoma cDNA library. Osteoclastomas are actively bone resorptive tumors, but are usually non-metastatic. In cryostat sections, osteoclastomas consist of ~30% multinucleated cells positive for tartrate resistant acid phosphatase (TRAP), a widely utilized phenotypic marker specific in vivo for osteoclasts (Minkin, *Calcif. Tissue Int.* 34:285-290 (1982)). The remaining cells are uncharacterized 'stromal' cells, a mixture of cell types with fibroblastic/mesenchymal morphology. Although it has not yet been definitively shown, it is generally held that the osteoclasts in these tumors are non-transformed, and are activated to resorb bone in vivo by substance(s) produced by the stromal cell element.

Monoclonal antibody reagents were used to partially characterize the surface phenotype of the multinucleated cells in the giant cell tumors of long bone. In frozen sections, all multinucleated cells expressed CD68, which has previously been reported to define an antigen specific for both osteoclasts and macrophages (Horton, M. A. and M. H. Helfrich, In *Biology and Physiology of the Osteoclast*, B. R. Rifkin and C. V. Gay, editors, CRC Press, Inc. Boca Raton, Fla., 33-54 (1992)). In contrast, no staining of giant cells was observed for CD11b or CD14 surface antigens, which are present on monocyte/macrophages and granulocytes (Arnaout, M. A. et al. *J. Cell. Physiol.* 137:305 (1988); Haziot, A. et al. *J. Immunol.* 141:547 (1988)). Cytochrome preparations of human peripheral blood monocytes were positive for CD68, CD11b, and CD14. These results demonstrate that the multinucleated giant cells of osteoclastomas have a phenotype which is distinct from that of macrophages, and which is consistent with that of osteoclasts.

Osteoclastoma tissue was snap frozen in liquid nitrogen and used to prepare poly A⁺ mRNA according to standard methods. cDNA cloning into a pcDNAII vector was carried out using a commercially-available kit (Librarian, In Vitrogen). Approximately 2.6×10⁶ clones were obtained, >95% of which contained inserts of an average length 0.6 kb.

Example 2—Stromal Cell mRNA Preparation

A portion of each osteoclastoma was snap frozen in liquid nitrogen for mRNA preparation. The remainder of the tumor was dissociated using brief trypsinization and mechanical disaggregation, and placed into tissue culture. These cells were expanded in Dulbecco's MEM (high glucose, Sigma) supplemented with 10% newborn calf serum (MA Bioproducts), gentamycin (0.5 mg/ml), l-glutamine (2 mM) and non-essential amino acids (0.1 mM) (Gibco). The stromal cell population was passaged at least five times, after which it showed a homogenous, fibroblastic looking cell population that contained no multinucleated cells. The stromal cells were mononuclear, tested negative acid phosphatase, and tested variably alkaline phosphatase positive. These findings indicate that propagated stromal cells (i.e., stromal cells that

are passaged in culture) are non-osteoclastic and non-activated.

Example 3—Identification of DNA Encoding Osteoclastoma-Specific or -Related Gene Products by Differential Screening of an Osteoclastoma cDNA Library

A total of 12,000 clones drawn from the osteoclastoma cDNA library were screened by differential hybridization, using mixed ^{32}P labelled cDNA probes derived from (1) giant cell tumor mRNA (stromal cell⁺, OC⁺), and (2) mRNA from stromal cells (stromal cell⁺, OC⁺) cultivated from the same tumor. The probes were labelled with ^{32}P dCTP by random priming to an activity of $-10^9\text{CPM}/\mu\text{g}$. Of these 12,000 clones, 195 gave a positive hybridization signal with giant cell (i.e., osteoclast and stromal cell) mRNA, but not with stromal cell mRNA. Additionally, these clones failed to hybridize to cDNA produced from mRNA derived from a variety of unrelated human cell types including epithelial cells, fibroblasts, lymphocytes, myelomonocytic cells, osteoblasts, and neuroblastoma cells. The failure of these clones to hybridize to cDNA produced from mRNA derived from other cell types supports the conclusion that these clones are either uniquely expressed in osteoclasts, or are osteoclast-related.

The osteoclast (OC) cDNA library was screened for differential hybridization to OC cDNA (stromal cell⁺, OC⁺) and stromal cell cDNA (stromal cell⁺, OC⁻) as follows:

NYTRAN filters (Schleicher & Schuell) were placed on agar plates containing growth medium and ampicillin. Individual bacterial colonies from the OC library were randomly picked and transferred, in triplicate, onto filters with pre-ruled grids and then onto a master agar plate. Up to 200 colonies were inoculated onto a single 90-mm filter/plate using these techniques. The plates were inverted and incubated at 37° C. until the bacterial inoculates had grown (on the filter) to a diameter of 0.5–1.0 mm.

The colonies were then lysed, and the DNA bound to the filters by first placing the filters on top of two pieces of Whatman 3 MM paper saturated with 0.5N NaOH for 5 minutes. The filters were neutralized by placing on two pieces of Whatman 3 MM paper saturated with 1M Tris-HCL, pH 8.0 for 3–5 minutes. Neutralization was followed by incubation on another set of Whatman 3 MM papers saturated with 1M Tris-HCL, pH 8.0/1.5M NaCl for 3–5 minutes. The filters were then washed briefly in 2×SSC.

DNA was immobilized on the filters by baking the filters at 80° C. for 30 minutes. Filters were best used immediately, but they could be stored for up to one week in a vacuum jar at room temperature.

Filters were prehybridized in 5–8 ml of hybridization solution per filter, for 2–4 hours in a heat sealable bag. An additional 2 ml of solution was added for each additional filter added to the hybridization bag. The hybridization

buffer consisted of 5×SSC, 5×Denhardt's solution, 1% SDS and 100 $\mu\text{g}/\text{ml}$ denatured heterologous DNA.

Prior to hybridization, labeled probe was denatured by heating in 1×SSC for 5 minutes at 100° C., then immediately chilled on ice. Denatured probe was added to the filters in hybridization solution, and the filters hybridized with continuous agitation for 12–20 hours at 65° C.

After hybridization, the filters were washed in 2×SSC/0.2% SDS at 50°–60° C. for 30 minutes, followed by washing in 0.2×SSC/0.2% SDS at 60° C. for 60 minutes.

The filters were then air dried and autoradiographed using an intensifying screen at -70° C. overnight.

Example 4—DNA Sequencing of Selected Clones

Clones reactive with the mixed tumor probe, but unreactive with the stromal cell probe, are expected to contain either osteoclast-related, or in vivo 'activated' stromal-cell-related gene products. One hundred and forty-four cDNA clones that hybridized to tumor cell cDNA, but not to stromal cell cDNA, were sequenced by the dideoxy chain termination method of Sanger et al. (Sanger F., et al. *Proc. Natl. Acad. Sci. USA* 74:5463 (1977)) using sequenase (US Biochemical). The DNASIS (Hitachi) program was used to carry out sequence analysis and a homology search in the GenBank/EMBL database.

Fourteen of the 195 tumor⁺ stromal⁻ clones were identified as containing inserts with a sequence identical to the osteoclast marker, type 5 tartrate-resistant acid phosphatase (TRAP) (GenBank accession number J04430 M19534). The high representation of TRAP positive clones also indicates the effectiveness of the screening procedure in enriching for clones which contain osteoclast-specific or related cDNA sequences.

Interestingly, an even larger proportion of the tumor⁺ stromal⁻ clones (77/195; 39.5%) were identified as human gelatinase B (macrophage-derived gelatinase) (Wilhelm, S. M. *J. Biol. Chem.* 264:17213 (1989)), again indicating high expression of this enzyme by osteoclasts. Twenty-five of the gelatinase B clones were identified by dideoxy sequence analysis; all 25 showed 100% sequence homology to the published gelatinase B sequence (Genbank accession number J05070). The portions of the gelatinase B cDNA sequence covered by these clones is shown in the FIGURE (SEQ ID NO: 33). An additional 52 gelatinase B clones were identified by reactivity with a ^{32}P -labelled probe for gelatinase B.

Thirteen of the sequenced clones yielded no readable sequence. A DNASIS search of GenBank/EMBL databases revealed that, of the remaining 91 clones, 32 clones contain novel sequences which have not yet been reported in the databases or in the literature. These partial sequences are presented in Table I. Note that three of these sequences were repeats, indicating fairly frequent representation of mRNA related to this sequence. The repeat sequences are indicated by^a, ^b superscripts (Clones 198B, 223B and 32C of Table I).

TABLE I

PARTIAL SEQUENCES OF 32 NOVEL OC-SPECIFIC OR -RELATED EXPRESSED GENES (cDNA CLONES)

34A (SEQ ID NO: 1)					
1 GCAATATCT	AAGTTTATTC	CTTGGATTC	TAOTGAGAGC	TGTTGAATT	GGTQATGTCA
61 AATGTTTCTA	GGGTTTTTTT	AGTTTGTTTT	TAITGAAAAA	TTTAAATTAT	TATGCTATAG
121 GTGATATCT	CTTTGAATAA	ACCTATAATA	GAAATAGCA	GCAGACAACA	
4B (SEQ ID NO: 2)					
1 GTGTCAACCT	GCATATCCTA	AAAATGTCAA	AATGCTGCAT	CTGGTTAATG	TCGGGGTAGG

TABLE I-continued

PARTIAL SEQUENCES OF 32 NOVEL OC-SPECIFIC OR -RELATED
EXPRESSED GENES (cDNA CLONES)

61 GGG					
12B (SEQ ID NO: 3)					
1 CTTCCTCTC	TTGCTTCCCT	TTCCCAAGCA	GAGGTGCTCA	CTCCATGGCC	ACCGCCACCA
61 CAGGCCACACA	GGGAGTACTG	CCAGACTACT	GCTGATGTC	TCTTAAGGCC	CAGGGAGTCT
121 CAACCAAGCTG	GTGGTGAATG	CTGCTGGCA	CGGGACCCCC	CCC	
28B (SEQ ID NO: 4)					
1 TTTTATTGT	AAATATATGT	ATTACATCCC	TAGAAAAAGA	ATCCCAGGAT	TTTCCCTCCT
61 GTGTGTTTT	GTCTTGCTTC	TTCATGGTCC	ATGATGCCAG	CTGAGGTTGT	CAGTACAATG
121 AAACCAAACT	GGCGGATGG	AAGCAGATTA	TTCGCAATT	TTTCCAGGTC	TTT
37B (SEQ ID NO: 5)					
1 GGCTGGACAT	GGGTGCCCTC	CACGTCCCTC	ATATCCOCAG	GCACACTCTG	GCCTCAGGTT
61 TTGCCCTGGC	CATGTCACT	ACCTGGAGTG	GGCCCTCCCCC	TTCTTCAGCC	TTGAATCAAA
121 AGCCACTTGG	TTAGGCGAGG	ATTCCACAGA	CCACTCATCA	CATTAACAAA	TATTTTGAAA
181 ACAAAAAAAA	AAAAAAA				
55B (SEQ ID NO: 6)					
1 TTGACAAAGC	TGTTTATTC	CACCAATAAA	TAGTATATGG	TGATTGGGGT	TTCTATTAT
61 AAGAGTAGTG	GCTATTATAT	GGGGTATCAT	GTGATGCTC	ATAAATAGTT	CATATCTACT
121 TAATTGCTT	TC				
60B (SEQ ID NO: 7)					
1 GAAGAGAGTT	GTATGTACAA	CCCAACAGG	CAAGGCAGCT	AAATGCAGAG	GGTACAGAGA
61 GATCCGAGG	GAATT				
86B (SEQ ID NO: 8)					
1 GGATGGAAAC	ATGTAGAAGT	CCAGAGAAAA	ACAATTTTAA	AAAAAGGTGG	AAAAGTTACG
61 GCAAACTGA	GATTCAGCA	TAAATCTTT	AGTTAGAAAT	GAGAGAAAGA	AGAGGGAGGC
121 TGGTTGCTGT	TGCACGTATC	AATAGGTTAT	C		
87B (SEQ ID NO: 9)					
1 TTCTTGATCT	TTAGAACACT	ATGAATAGGG	AAAAAGAAAA	AAACTGTTCA	AAATAAAATG
61 TAGGAGCCGT	GCCTTTGGA	TGCTTGAGTG	AGGAGCTCAA	CAAGTCCCTCT	CCCAAGAAAG
181 CAATGATAAA	ACTTGACAAA	A			
98B (SEQ ID NO: 10)					
1 ACCCATTTCT	AACAATTTT	ACTGIAAAAT	TTTTGGTCAA	AGTTCTAAGC	TAAATCACAT
61 CTCAAAGAAT	AGAGGCAATA	TATAGCCCAT	CTTACTAGAC	ATACAGTATT	AAACTGGACT
121 GAATATGAGG	ACAAGCTCTA	GTGGTCATTA	AAACCCCTCAG	AA	
110B (SEQ ID NO: 11)					
1 ACATATATTA	ACAGCATTC	TTTGGCCAAA	ATCTACAGT	TTGTAGAATC	CTACTGTATA
61 TAAAGTGCGA	ATGTATCAAG	TATAGACTAT	GAAAGTGCAA	ATAACAAATC	AAGGTTAGAT
121 TAACTTTTTT	TTTTTACATT	ATAAAATTA	CTTGT		
118B (SEQ ID NO: 12)					
1 CCAAATTTCT	CTGGAATCCA	TCCTCCCTCC	CATCACCATA	GCCTCGAGAC	GTCAATTTCTG
61 TTTGACTACT	CCAGC				
133B (SEQ ID NO: 13)					
1 AACTAACCTC	CTCGGACCCC	TGCTCACTC	ATTACACCA	ACCAOCCAAC	TATCTATAAA
61 CCTGAGCCAT	GGCCATCCCT	TATGAGCCGC	GCAGTGATTA	TAGGCTTTCTG	CTCTAAGATA
121 AAAT					
140B (SEQ ID NO: 14)					
1 ATTATTATTC	TTTTTTTATG	TTAGCTTAGC	CATGCAAAAT	TTACTGGTGA	AGCAGTTAAT
61 AAAACACACA	TCCCATTGAA	GGGTTTGTGA	CAITTCAGTC	CTTACAAATA	ACAAAGCAAT
121 GATAAACCCG	GCACGTCCTG	ATAGGAAATT	C		
144B (SEQ ID NO: 15)					
1 CGTGACACAA	ACATGCATTC	GTTTTATTC	TAAACAGCC	TGGTTTCCTA	AAACAATACA
61 AACAGCATGT	TCATCAGCAG	GAAGCTGGCC	GTGGCCAGGG	GGGCC	
198B* (SEQ ID NO: 16)					
1 ATAGGTTAGA	TTCTCATTC	CGGGACTAGT	TAGCTTTAAG	CACCTAGAG	GACTAGGGTA
61 ATCTGACTTC	TCATTTCTTA	AGTTCCCTCT	TATATCTCA	AGGTAGAAAT	GTCTATGTTT
121 TCTACTCCAA	TTTCAAAATC	TATTCATAAG	TCTTTGGTAC	AAGTTACATG	ATAAAAAAGAA
181 ATGTGATTTG	TCTTCCCTTC	TTTGCACCTT	TRAAATAAAG	TATTTATCTC	CTGTCTACAG
241 TTTAAT					
212B (SEQ ID NO: 17)					
1 GTCCAGTATA	AAGGAAAGCG	TAAAGTCGGT	AAGCTAGAGG	ATTGTAATA	TCTTTTATGT
61 CCTCTAGATA	AAACACCCGA	TAAACAGATG	TAAACCTTTT	ATGTTTTGAT	TTGCTTTAAA
121 AATGGCCCTC	TACACATTAG	CTCCAGCTAA	AAAGACACAT	TGAGAGCTTA	GAGGATAGTC
181 TCTGGAGC					
223B* (SEQ ID NO: 18)					
1 GCACCTTGGA	GGGAGTTGGT	GTGCTATTT	TGAAGCAGAT	GTGGTGATAC	TGAQATTGTC
61 TGTTCAGTTT	CCCCATTTGT	TTGTGCTTCA	AATGATCCTT	CCTACTTTGC	TTCTCTCCAC
121 CCATGACCTT	TTCACTGTG	GCCATCAAGG	ACITTCCTGA	CAGCTTGTGT	ACTCTTAGGC
181 TAAGAGATGT	GACTACAGCC	TGCCCTGAC	TG		
241B (SEQ ID NO: 19)					
1 TGTTAGTTTT	TAGGAAGGCC	TGTCTTCTGG	GAGTGAGGTT	TATTAGTCCA	CTTCTTGGAG
61 CTAGACGTCC	TATAGTTAGT	CACCTGGGGAT	GGTGAAAGAG	GGAGAAAGAG	AAGGGCGAAG
121 GGAAGGGCTC	TTTGCTAGTA	TCTCCATTTC	TAGAAGATGG	TTTAGATGAT	AACCAAGGTT
181 CTATATGAGC	ATAGTAAGGC	TGT			
32C* (SEQ ID NO: 20)					
1 CCTATTCTG	ATCCTGACTT	TGGACAAGGC	CCTTCAGCCA	GAAGACTGAC	AAAGTCATCC
121 TCCGTCTACC	AGAGCGTGCA	CTTGATGATC	TAAATAAAGC	TTCATCTCCG	GCTGTGCCTT
161 GGGTGAAGG	GGCAGGATTC	TGCAGCTGCT	TTTGCATTTC	TCTTCTTAAA	TTTCAAT

TABLE I-continued

PARTIAL SEQUENCES OF 32 NOVEL OC-SPECIFIC OR -RELATED EXPRESSED GENES (cDNA CLONES)					
34C (SEQ ID NO: 21)	GTGTGTTTAT	TCCTGTACAA	ATCATTACAA	AACCAAGTCT	GGGGCAGTCA
1 CCGAGCGTAG	CCATCACCCC	AGTGCAATGG	CTAGCTGCTG	GCCTTT	
61 CCGCCCCCAC					
47C (SEQ ID NO: 22)	CAAAGCAGGC	AACCCCTTT	GGCACTGCTG	CCACTGGGGT	CATGGGGTT
1 TTAGTTCAGT	GGGAGGTTTC	CCCAACACCC	TCCTCTGCTT	CCCTGTGTGT	CGGGGTCTCA
61 GTGGCAGCTG	CAGAGTGGG				
121 GGAGCTGACC					
65C (SEQ ID NO: 23)	TAAGAGAGAT	TTTGGTCTTA	AAGGCTTCAT	CATGAAAGTG	TACATGCATA
1 GCTGAATGTT	AATACGTGG	TATGGATGGT	TGCTTGTTTA	TTAACTAAAG	ATGTACAGCA
61 TGCAAGTGTG	TTAGAGTCTT	CTTAATATTG	ATGTCCTAAC	ACTGGGTCTG	CTTATGC
121 AACTGCCCGT					
79C (SEQ ID NO: 24)	TATGGAATCC	AGAAGGGAAA	CAAGCACTGG	ATAATTAATA	ACAGCTGGGG
1 GGCACTGGGA	GGAAACAAAG	GATATATCT	CATGGCTCGA	AATAAGAAAC	AOCCTGTGG
61 AGAAAACTGG	CTGGCCAGCT	TCCCAAGAT	GTGACTCCAG	CCAGAAA	
121 CATTGCCAAC					
84C (SEQ ID NO: 25)	ACCGTCTTTA	TTCTCTCCT	GCCTCAGAGG	TCAGGAAGGA	GGTCTGGCAG
1 GCCAGGGCGG	GGGCGCTAGT	CATCTGTGGC	AGCGAAGGTG	AAGGGACTCA	CCTTGTGCCC
61 GACCTGCAGT	TAGAACTTGT	TCTGGAATTC	C		
121 CGTGCCCTGAG					
86C (SEQ ID NO: 26)	CACTCTGGTA	TTTTTAGTIT	AACAATATAT	GTGTTGTGTC	TTGGAAAITA
1 AACTCTTTCA	ATTCATATTG	AGCTGTCTCA	TTCTTTTTTT	AATGGTCATA	TACAGTAGTA
61 GTTCATATCA	AGAATATATC	CTAATACTTT	TAAAA		
121 TTCAATTATA					
87C (SEQ ID NO: 27)	GAAGGCCTGA	GGCCTAGGGG	CCGRGGCTGG	CCTGCGTCTC	AGTCTGGGA
1 GGATAAGAAA	CGCAGAGGTT	GAGAGGGGCA	CTTCCTCTTG	CTTAGGTTGG	TGAGGATCTG
61 CGCAGCAGCC	GCCGCTGGAG	AGCCACAAAA			
121 GTCCTGGTTG					
88C (SEQ ID NO: 28)	AGAGTTTGAC	CTGGAGCCCG	ATACCTACTG	CCGCTATGAC	TCGGTCAGCG
1 CTGACCTTGG	AGCCGTGAGC	GACGACTCCG	GTGGGGAAGT	TCTGCGGCGA	T
61 TGTTCACCG					
89C (SEQ ID NO: 29)	GTGGATAGTG	CTTTGTGTGA	GCAAATGCTC	CCTCCTTAAG	GTTATAGGGC
1 ATCCCTGGCT	TGOGAGTGTG	GAAGTACTAC	TTAACTGTCT	GTCTGCTTG	GCTGTGTGTA
61 TCCTGAGTGT	GTGATGTTGT	GCTAACATA	AGAATAC		
121 TCGTTTTCTG					
101C (SEQ ID NO: 30)	CCCTCTCCTC	CTCCATCCCC	ATACATCACC	AGGTCTAATG	TTTACAAACG
1 GGCTGGGCAT	GGCTCTGAAG	CCAAGGGCCG	TCCGTGCCAC	GGTGGCTGTG	AGTATTCCTC
61 GTGCCAGCCC	CCCATAGGT	TGGAGTATCT	GC		
121 CGTAGCTTT					
112C (SEQ ID NO: 31)	COGCGATACA	GACCCACAGA	GTGCCATCCC	TGAGAGACCA	GACCGCTCCC
1 CCAACTCCTA	CTAAATAAAA	CATGAAGCAC			
161 CAATACTCTC					
114C (SEQ ID NO: 32)	TGTCCTCATGG	TGGGAAGGAA	CATGGTACAT	TTC	
1 CATGGATGAA					

*Repeated 3 times

*Repeated 2 times

Sequence analysis of the OC⁺ stromal cell⁻ cloned DNA sequences revealed, in addition to the novel sequences, a number of previously-described genes. The known genes identified (including type 5 acid phosphatase, gelatinase B, cystatin C (13 clones), Alu repeat sequences (11 clones), creatine kinase (6 clones) and others) are summarized in Table II. In situ hybridization (described below) directly demonstrated that gelatinase B mRNA is expressed in multinucleated osteoclasts and not in stromal cells. Although gelatinase B is a well-characterized protease, its expression at high levels in osteoclasts has not been previously described. The expression in osteoclasts of cystatin C, a cysteine protease inhibitor, is also unexpected. This finding has not yet been confirmed by in situ hybridization. Taken together, these results demonstrate that most of these identified genes are osteoclast-expressed, thereby confirming the effectiveness of the differential screening strategy for identifying DNA encoding osteoclast-specific or -related gene products. Therefore, novel genes identified by this method have a high probability of being OC-specific or related.

In addition, a minority of the genes identified by this screen are probably not expressed by OCs (Table II). For example, type III collagen (6 clones), collagen type I (1 clone), dermatansulfate (1 clone), and type VI collagen (1

clone) are more likely to originate from the stromal cells or from osteoblastic cells which are present in the tumor. These cDNA sequences survive the differential screening process either because the cells which produce them in the tumor in vivo die out during the stromal cell propagation phase, or because they stop producing their product in vitro. These clones do not constitute more than 5-10% of the all sequences selected by differential hybridization.

TABLE II

SEQUENCE ANALYSIS OF CLONES ENCODING KNOWN SEQUENCES FROM AN OSTEOCLASTOMA cDNA LIBRARY	
Clones with Sequence Homology to Collagenase Type IV	25 total
Clones with Sequence Homology to Type 5 Tetrakis Resistant Acid Phosphatase	14 total
Clones with Sequence Homology to Cystatin C	13 total
Clones with Sequence Homology to Alu-repeat Sequences	11 total
Clones with Sequence Homology to Creatine Kinase	6 total
Clones with Sequence Homology to	6 total

TABLE II-continued

SEQUENCE ANALYSIS OF CLONES ENCODING KNOWN SEQUENCES FROM AN OSTEOCLASTOMA cDNA LIBRARY	
Type III Collagen	
Clones with Sequence Homology to MHC Class I γ Invariant Chain	5 total
Clones with Sequence Homology to MHC Class II β Chain	3 total
One or Two Clone(s) with Sequence Homology to Each of the Following:	10 total
$\alpha 1$ collagen type I	
γ interferon inducible protein	
osteopontin	
Human chondroitin/dermatansulfate	
α globin	
β glucosidase/sphingolipid activator	
Human CAP1 protein (Ca binding)	
Human EST 01024	
Type VI collagen	
Human EST 00553	

Example 5—In situ Hybridization of OC-Expressed Genes

In situ hybridization was performed using probes derived from novel cloned sequences in order to determine whether the novel putative OC-specific or -related genes are differentially expressed in osteoclasts (and not expressed in the stromal cells) of human giant cell tumors. Initially, in situ hybridization was performed using antisense (positive) and sense (negative control) cRNA probes against human type IV collagenase/gelatinase B labelled with 35 S-UTP.

A thin section of human giant cell tumor reacted with the antisense probe resulted in intense labelling of all OCs, as indicated by the deposition of silver grains over these cells, but failed to label the stromal cell elements. In contrast, only minimal background labelling was observed with the sense (negative control) probe. This result confirmed that gelatinase B is expressed in human OCs.

In situ hybridization was then carried out using cRNA probes derived from 11/32 novel genes, labelled with digoxigenin UTP according to known methods.

The results of this analysis are summarized in Table III. Clones 28B, 118B, 140B, 198B, and 212B all gave positive reactions with OCs in frozen sections of a giant cell tumor, as did the positive control gelatinase B. These novel clones therefore are expressed in OCs and fulfill all criteria for OC-relatedness. 198B is repeated three times, indicating relatively high expression. Clones 4B, 37B, 88C and 98B produced positive reactions with the tumor tissue; however the signal was not well-localized to OCs. These clones are therefore not likely to be useful and are eliminated from further consideration. Clones 86B and 87B failed to give a positive reaction with any cell type, possibly indicating very low level expression. This group of clones could still be useful but may be difficult to study further. The results of this analysis show that 5/11 novel genes are expressed in OCs, indicating that ~50% of novel sequences likely to be OC-related.

To generate probes for the in situ hybridizations, cDNA derived from novel cloned osteoclast-specific or -related cDNA was subcloned into a BlueScript II SK(–) vector. The orientation of cloned inserts was determined by restriction analysis of subclones. The T7 and T3 promoters in the BlueScriptII vector was used to generate 35 S-labelled (35 S-UTP 850 Ci/mmol, Amersham, Arlington Heights, Ill.), or

UTP digoxigenin labelled cRNA probes.

TABLE III

In Situ HYBRIDIZATION USING PROBES DERIVED FROM NOVEL SEQUENCES			
Clone	Reactivity with:		
	Osteoclasts	Stromal Cells	
4B	+	+	
28B*	+	–	
37B	+	+	
86B	–	–	
87B	–	–	
88C	+	+	
98B	+	+	
118B*	+	–	
140B*	+	–	
198B*	+	–	
212B*	+	–	
Gelatinase B*	+	–	

*OC-expressed, as indicated by reactivity with antisense probe and lack of reactivity with sense probe on OCs only.

In situ hybridization was carried out on 7 micron cryostat sections of a human osteoclastoma as described previously (Chang, L.-C. et al. *Cancer Res.* 49:6700 (1989)). Briefly, tissue was fixed in 4% paraformaldehyde and embedded in OCT (Miles Inc., Kankakee, Ill.). The sections were rehydrated, postfixed in 4% paraformaldehyde, washed, and pretreated with 10 mM DTT, 10 mM iodoacetamide, 10 mM N-ethylmaleimide and 0.1 triethanolamine-HCL. Prehybridization was done with 50% deionized formamide, 10 mM Tris-HCl, pH 7.0, 1x Denhardt's, 500 mg/ml tRNA, 80 mg/ml salmon sperm DNA, 0.3M NaCl, mM EDTA, and 100 mM DTT at 45° C. for 2 hours. Fresh hybridization solution containing 10% dextran sulfate and 1.5 ng/ml 35 S-labelled or digoxigenin labelled RNA probe was applied after heat denaturation. Sections were coverslipped and then incubated in a moistened chamber at 45°–50° C. overnight. Hybridized sections were washed four times with 50% formamide, 2x SSC, containing 10 mM DTT and 0.5% Triton X-100 at 45° C. Sections were treated with RNase A and RNase T1 to digest single-stranded RNA, washed four times in 2x SSC/10 mM DTT.

In order to detect 35 S-labelling by autoradiography, slides were dehydrated, dried, and coated with Kodak NTB-2 emulsion. The duplicate slides were split, and each set was placed in a black box with desiccant, sealed, and incubated at 4° C. for 2 days. The slides were developed (4 minutes) and fixed (5 minutes) using Kodak developer D19 and Kodak fixer. Hematoxylin and eosin were used as counterstains.

In order to detect digoxigenin-labelled probes, a Nucleic Acid Detection Kit (Boehringer-Mannheim, Cat. #1175041) was used. Slides were washed in Buffer 1 consisting of 100 mM Tris/150 mM NaCl, pH7.5, for 1 minute. 100 μ l Buffer 2 was added (made by adding 2 mg/ml blocking reagent as provided by the manufacturer) in Buffer 1 to each slide. The slides were placed on a shaker and gently swirled at 20° C.

Antibody solutions were diluted 1:100 with Buffer 2 (as provided by the manufacturer). 100 μ l of diluted antibody solution was applied to the slides and the slides were then incubated in a chamber for 1 hour at room temperature. The slides were monitored to avoid drying. After incubation with antibody solution, slides were washed in Buffer 1 for 10 minutes, then washed in Buffer 3 containing 2 mM levamisole for 2 minutes.

After washing, 100 μ l color solution was added to the slides. Color solution consisted of nitroblue/tetrazolium salt

13

(NBT) (1:225 dilution) 4.5 μ l, 5-bromo-4-chloro-3-indolyl phosphate (1:285 dilution) 3.5 μ l, levamisole 0.2 mg in Buffer 3 (as provided by the manufacturer) in a total volume of 1 ml. Color solution was prepared immediately before use.

After adding the color solution, the slides were placed in a dark, humidified chamber at 20° C. for 2-5 hours and monitored for color development. The color reaction was stopped by rinsing slides in TE Buffer.

The slides were stained for 60 seconds in 0.25% methyl green, washed with tap water, then mounted with water-based Permout (Fisher).

Example 6—Immunohistochemistry

Immunohistochemical staining was performed on frozen and paraffin embedded tissues as well as on cytospin preparations (see Table IV). The following antibodies were used: polyclonal rabbit anti-human gelatinase antibodies; Ab110 for gelatinase B; monoclonal mouse anti-human CD68 antibody (clone KP1) (DAKO, Denmark); Mol (anti-CD11b) and Mo2 (anti-CD14) derived from ATCC cell lines HB CRL 8026 and TIB 228/HB44. The anti-human gelatinase B antibody Ab110 was raised against a synthetic peptide with the amino acid sequence EALMYPMYRFTEGPPLHK (SEQ ID NO: 34), which is specific for human gelatinase B (Corcoran, M. L. et al. *J. Biol. Chem.* 267:515 (1992)).

Detection of the immunohistochemical staining was achieved by using a goat anti-rabbit glucose oxidase kit (Vector Laboratories, Burlingame Calif.) according to the manufacturer's directions. Briefly, the sections were rehydrated and pretested with either acetone or 0.1% trypsin. Normal goat serum was used to block nonspecific binding. Incubation with the primary antibody for 2 hours or overnight (Ab110:1/500 dilution) was followed by either a glucose oxidase labeled secondary anti-rabbit serum, or, in the case of the mouse monoclonal antibodies, were reacted with purified rabbit anti-mouse Ig before incubation with the secondary antibody.

Paraffin embedded and frozen sections from osteoclastomas (GCT) were reacted with a rabbit antiserum against gelatinase B (antibody 110) (Corcoran, M. L. et al. *J. Biol. Chem.* 267:515 (1992)), followed by color development with glucose oxidase linked reagents. The osteoclasts of a giant cell tumor were uniformly strongly positive for gelatinase B, whereas the stromal cells were unreactive. Control sections reacted with rabbit preimmune serum were negative. Identical findings were obtained for all 8 long bone giant cell tumors tested (Table IV). The osteoclasts present in three out of four central giant cell granulomas (GCG) of the mandible were also positive for gelatinase B expression. These neoplasms are similar but not identical to the long bone giant cell tumors, apart from their location in the jaws (Shafer, W. G. et al., *Textbook of Oral Pathology*, W. B. Saunders Company, Philadelphia, pp. 144-149 (1983)). In contrast, the multinucleated cells from a peripheral giant cell tumor, which is a generally non-resorptive tumor of oral soft tissue,

14

were unreactive with antibody (Shafer, W. G. et al., *Textbook of Oral Pathology*, W. B. Saunders Company, Philadelphia, pp. 144-149 (1983)).

Antibody 110 was also utilized to assess the presence of gelatinase B in normal bone (n=3) and in Paget's disease, in which there is elevated bone remodeling and increased osteoclastic activity. Strong staining for gelatinase B was observed in osteoclasts both in normal bone (mandible of a 2 year old), and in Paget's disease. Staining was again absent in controls incubated with preimmune serum. Osteoblasts did not stain in any of the tissue sections, indicating that gelatinase B expression is limited to osteoclasts in bone. Finally, peripheral blood monocytes were also reactive with antibody 110 (Table IV).

TABLE IV

DISTRIBUTION OF GELATINASE B IN VARIOUS TISSUES

Samples	Antibodies tested Ab 110 gelatinase B
GCT frozen (n = 2)	
giant cells	+
stromal cells	-
GCT paraffin (n = 6)	
giant cells	+
stromal cells	-
central GCG (n = 4)	
giant cells	+(34)
stromal cells	-
peripheral GCT (n = 4)	
giant cells	-
stromal cells	-
Paget's disease (n = 1)	
osteoclasts	+
osteoblasts	-
normal bone (n = 3)	
osteoclasts	+
osteoblasts	-
monocytes (cytospin)	+

Distribution of gelatinase B in multinucleated giant cells, osteoclasts, osteoblasts and stromal cells in various tissues. In general, paraffin embedded tissues were used for these experiments; exceptions are indicated.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(111) NUMBER OF SEQUENCES: 34

-continued

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 170 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCAAAATATCT AAGTTTATTG CTTGQATTTT TAGTGAGAGC TGTGAATTT GGTGATGTCA 60
 AATGTTTCTA GGGTTTTTTT AGTTTGTITT TATTGAAAAA TTTAATTATT TATGCTATAG 120
 GTGATATTCT CTTTGAATAA ACCTATAATA GAAAAAGCA GCAGACAACA 170

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 63 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGTCAACCT GCATATCCTA AAAATGTCAA AATGCTGCAT CTGGTTAATG TCGGGGTAGG 60
 GGG 63

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 163 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTTCCCTCTC TTGCTTCCCT TTCCCAAGCA GAGGTGCTCA CTCCATGCC ACCGCCACCA 60
 CAGGCCACCA GGGAGTACTG CCAGACTACT GCTGATGTTT TCTTAAGGCC CAAGGAGTCT 120
 CAACCAAGCTG GTGGTGAATG GTGCCTGGCA CGGGACCCCC CCC 163

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 173 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTTTATTTGT AAATATATGT ATTACATCCC TAGAAAAAGA ATCCCAGGAT TTTCCCTCCT 60
 GTGTGTTTTT GTCTTGCTTC TTCATGGTCC ATGATGCCAG CTGAGGTTGT CAGTACAATG 120
 AAACCAAACCT GGCAGGATGG AAGCAGATTA TTCTGCCATT TTTCCAGGTC TTT 173

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 197 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double

-continued

(D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (genomic)

(1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

GGCTGACAT GGGTGCCCTC CACGTCCCTC ATATCCCCAG GCACACTCTG GCCTCAGGTT      60
TTGCCCTGGC CATGTCATCT ACCTGGAGTG GGCCTCCCC TTCTTCAGCC TTGAATCAAA      120
AGCCACTTTG TTAGGCGAGG ATTTCCAGA CCACTCATCA CATTAAAAAA TATTTTAAAA      180
ACAAAAAAA AAAAAAA      197

```

(2) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 132 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (genomic)

(1) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

TTGACAAAGC TGTTTATTTT CACCAATAAA TAGTATATGG TGATTGGGGT TTCTATTTAT      60
AAGAGTAGTG GCTATTATAT GGGGTATCAT GTTGATGCTC ATAAATAGTT CATATCTACT      120
TAATTTGCCT TC      132

```

(2) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (genomic)

(1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

GAAGAGAGTT GTATGTACAA CCCCAACAGG CAAGGCAGCT AAATGCAGAG GGTACAGAGA      60
GATCCCGAGG GAATT      75

```

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 151 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (genomic)

(1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

GGATGGAAAC ATGTAGAAAT CCAGAGAAAA ACAATTTTAA AAAAAAGGTGG AAAAGTTACG      60
GCAAACTGA GATTTAGCA TAAATCTTT AGTTAGAAGT GAGAGAAAGA AGAGGGAGGC      120
TGOTTGCTGT TGACGTATC AATAGTTAT C      151

```

(2) INFORMATION FOR SEQ ID NO:9:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 141 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (genomic)

-continued

(1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

TTCTTGATCT TTAGAACACT ATGAATAAGG AAAAAAGAAA AAAGTGTTC AATAAAAAATG      60
TAGGAGCCGT GCTTTTGGAA TGCTTGAGTG AGGAGCTCAA CAAGTCCTCT CCCAAGAAAG      120
CAATGATAAA ACTTGACAAA A                                     141

```

(2) INFORMATION FOR SEQ ID NO:10:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 162 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(1.1) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

ACCCATTTCT AACAAATTTT ACTGTAAAAAT TTTTGOTCAA AGTTCTAAGC TTAATCACAT      60
CTCAAAGAAT AGAGGCAATA TATAGCCCAT CTTACTAGAC ATACAOTATT AAAGTGGACT      120
GAATATGAGG ACAAGCTCTA GTGOTCATT AACCCTCAG AA                               162

```

(2) INFORMATION FOR SEQ ID NO:11:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 157 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(1.1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

ACATATATTA ACAGCATTCA TTTGGCCAAA ATCTACACGT TTGTAGAATC CTACTGTATA      60
TAAAGTGGGA ATGTATCAAG TATAGACTAT GAAAGTGCAA ATAACAAATC AAGGTTAGAT      120
TAACTTTTTT TTTTACATT ATAAAATTAA CTTGTTT                               157

```

(2) INFORMATION FOR SEQ ID NO:12:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(1.1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

CCAAATTTCT CTGGAATCCA TCCTCCCTCC CATCACCATA GCCTCGAGAC GTCATTTCTG      60
TTTGACTACT CCAGC                                     75

```

(2) INFORMATION FOR SEQ ID NO:13:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 124 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(1.1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

AACTAACCTC CTCGACCCC TGCCTCACTC ATTTACACCA ACCACCCAAC TATCTATAAA      60
CCTGAGCCAT GGCCATCCCT TATGAGCGGC GCAGTGATTA TAGGCTTTTC CTCTAAGATA      120

```

-continued

AAAT

124

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 151 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

ATTATTATTC TTTTATTATG TTAGCTTAGC CATGCAAAAT TTAAGTGTGA AGCAGTTAAT      60
AAAACACACA TCCCATTTGAA GGGTTTGTGA CATTTCAGTC CTTACAAATA ACAAAGCAAT      120
GATAAAGCCG GCACGTCCTG ATAGGAAATT C                                     151

```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 105 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

CGTGACACAA ACATGCATTC GTTTTATTCA TAAACAGCC TGGTTTCCTA AAACAATACA      60
AACAGCATGT TCATCAACAG GAAAGTGGCC GTGGGCAGGG GGGGCC                                     105

```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 246 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

ATAAGTTAGA TTCTCATTCA CCGGACTAGT TAGCTTTAAG CACCCTAGAG GACTAGGGTA      60
ATCTGACTTC TCATTTCCTA AGTTCCCTCT TATATCCTCA AGGTAGAAAT GTCTATGTTT      120
TCTACTCCAA TTCATAAATC TATTCATAAG TCTTTGGTAC AAGTTACATG ATAAAAAGAA      180
ATGTGATTTG TCTTCCCTTC TTTGCACTTT TGAAATAAAG TATTTATCTC CTGTCTACAG      240
TTTAAT                                     246

```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 188 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```

GTCCAGTATA AAGGAAAGCG TTAAGTCGGT AAGCTAGAGG ATTGTAAATA TCTTTTATGT      60
CCTCTAGATA AAACACCCGA TTAACAGATG TTAACCTTTT ATGTTTGTAT TTGCTTTAAA      120
AATGGCCTTC TACACATTAG CTCCAGCTAA AAAGACACAT TGAGAGCTTA GAGGATAGTC      180

```

-continued

TCTGGAGC

188

(2) INFORMATION FOR SEQ ID NO:18:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 212 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(2.1) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCACTTGGAA	OGGAGTTGOT	GTGCTATTTT	TGAAACAGAT	GTGOTOATAC	TGAGATTGTC	60
TGTTCAGTTT	CCCCATTGT	TTGTGCTTCA	AATGATCCTT	CCTACTTTGC	TTCTCTCCAC	120
CCATOACCTT	TTTCACTGTG	GCCATCAAGG	ACTTTCCTGA	CAGCTTGTTG	ACTCTTAGGC	180
TAAGAGATGT	GACTACAGCC	TGCCCTGAC	TO			212

(2) INFORMATION FOR SEQ ID NO:19:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 203 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(2.1) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGTTAGTTTT	TAGGAAGGCC	TOTCTTCTGG	GAGTGAGGTT	TATTAGTCCA	CTTCTTOGAG	60
CTAGACGTCC	TATAGTTAGT	CACTGGGGAT	GGTGAAAGAG	GGAGAAGAGG	AAGGGCGAAG	120
GGAAGGGCTC	TTTGCTAGTA	TCTCCATTTC	TAGAAGATGG	TTTAGATGAT	AACCACAGGT	180
CTATATGAGC	ATAOTAAGGC	TGT				203

(2) INFORMATION FOR SEQ ID NO:20:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 177 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(2.1) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCTATTTCTG	ATCCTGACTT	TGGACAAGGC	CCTTCAGCCA	GAAGACTGAC	AAAGTCATCC	60
TCCOTCTACC	AGAGCGTGCA	CTTGATGATCC	TAAATAAGC	TTTATCTCCG	GCTGTGCCTT	120
GGGTGGAAGG	GGCAGGATTC	TGCAGCTGCT	TTTGCAATTC	TCTTCCTAAA	TTTCATT	177

(2) INFORMATION FOR SEQ ID NO:21:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 106 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(1.1) MOLECULE TYPE: DNA (genomic)

(2.1) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGGAGCGTAG	GTGTGTTTAT	TCCTGTACAA	ATCATTACAA	AACCAAGTCT	GGGGCAOTCA	60
CCCCCCCAC	CCATCACCCC	AGTGCAATGG	CTAGCTGCTG	GCCTTT		106

-continued

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 139 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TTAGTTCAAT CAAAGCAAGC AACCCCTTT GGCAGTCTG CCACTGGGGT CATGGCGTT      60
GTGGCAGCTG GGGAGGTTTC CCAACACCC TCCTCTGCTT CCCTGTGTGT CGGGGTCTCA    120
GGAGCTGACC CAGAGTGA      139
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 177 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
GCTGAATGTT TAAGAGAGAT TTGGTCTTA AAGGCTTCAT CATGAAAGTG TACATGCATA      60
TGCAAGTGTG AATTACGTGG TATGGATGGT TGCTTGTTTA TTAATAAAG ATGTACAGCA    120
AACTGCCCGT TTAGAGTCTT CTTAATATTG ATGTCCTAAC ACTGGGTCTG CTTATGC      177
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
GGCAGTGGGA TATGGAATCC AGAAGGGAAA CAAAGCACTGG ATAATTAAAA ACAAGTGGGG      60
AGAAAAGTGG GGAACAAAG GATATATCCT CATGGCTCGA AATAAGAACA ACGCCTGTGG    120
CATTGCCAAC CTGGCCAGCT TCCCAAGAT GTGACTCCAG CCAGAAA      167
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 151 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
GCCAGGGCGG ACCGTCTTTA TTCCTCTCCT GCCTCAGAGG TCAGGAAGGA GGTCTGGCAG      60
GACCTGCAGT GGGCCCTAGT CATCTGTGGC AGCGAAGGTG AAGGGACTCA CTTGTCCGC    120
COTGCCTGAG TAAACTTGT TCTGGAATTC C      151
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 156 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AACCTCTTCA CACTCTGTGA TTTTATGTTT AACAAATATAT GTGTGTGTGC TTGGAAATTA	60
GTTTCATATCA ATTCATATTG AGCTGTCTCA TTCTTTTTTT AATGGTCATA TACAGTAGTA	120
TTCAATTATA AGAATATATC CTAATACTTT TAAAAA	156

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 150 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGATAAGAAA GAAGGCCTGA GGGCTAGGGG CCGGGGCTGG CCTGCGTCTC AGTCCTGGGA	60
CGCAGCAGCC CGCACAGGTT GAGAGGGGCA CTTCCTCTTG CTTAGGTTGG TGAGGATCTG	120
GTCTTGTTG GCGGTTGGAG AGCCACAAAA	150

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 212 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCACCTGGAA GGGAGTTGGT GTGCTATTTT TGAAGCAGAT GTGGTGATAC TGAGATTGTC	60
TGTTACAGTT CCCCATTTGT TTGTGCTTCA AATGATCCTT CCTACTTTGC TTCTCTCCAC	120
CCATGACCTT TTTCACGTG GCCATCAAGG ACTTTCCTGA CAGCTTGTGT ACTCTTAGGC	180
TAAGAGATGT GACTACAGCC TGCCCTOAC TG	212

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 157 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATCCCTGGCT GTGGATAGTG CTTTGTGTGA GCAAATGCTC CCTCCTTAAG GTTATAGGGC	60
TCCCTGAGTT TGGGAGTGTG GAAGTACTAC TTAAGTGTCT GTCTGCTTG GCTGTCGTTA	120
TCOTTTTCTG GTGATGTTGT OCTAACAATA AGAATAC	157

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 152 base pairs
 (B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGCTGGGCAT CCCTCTCCTC CTCCATCCCC ATACATCACC AGGTCTAATG TTTACAAACG	60
GTOCCAGCCC GGCTCTGAAG CCAAGGGCCG TCCGTGCCAC GGTGGCTGTG AGTATTCCTC	120
CGTTAOCITT CCCATAAGGT TGGAGTATCT GC	152

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CCAACTCCTA CCGGATACA GACCCACAGA GTGCCATCCC TGAGAGACCA GACCGCTCCC	60
CAATACTCTC CTAAAATAAA CATGAAGCAC	90

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CATGGATGAA TGTCTCATGG TGGGAAGGAA CATGATACAT TTC	43
---	----

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2333 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AGACACCTCT GCCCTACCA TGAGCCTCTG GCAGCCCCCTG GTCCTGGTGC TCCTGGTCT	60
GGGCTGCTGC TTTGCTGCCC CCAGACAAGC CCAGTCCACC CTTGTCTCT TCCCTGGAGA	120
CCTGAGAACC AATCTCACC ACAGGCAGCT GGCAGAGGAA TACCTGTACC OCTATGTTA	180
CACTCGGGTG GCAGAGATGC GTGAGAGATC GAAATCTCTG GGGCCTGCGC TGCTGCTTCT	240
CCAGAAGCAA CTOTCCCTGC CCGAGACCGG TGAAGTGGAT AGCGCCACGC TGAAAGCCAT	300
GCGAACCCCA CGGTGCGGGG TCCCAGACCT GGGCAGATTC CAAACCTTTG AGGGCGACCT	360
CAAATGGCAC CACCACAACA TCACCTATTG GATCCAAAAC TACTCGGAAG ACTTGCCGCG	420
GGCGGTGATT GACGACGCTT TTGCCCCGCG CTTGCGACTG TGGAGCGCGG TGACGCCGCT	480
CACCTTCACT CCGGTGTACA GCCGGGACGC AGACATCGTC ATCCAGTTTG GTGTCGCGGA	540
GCACGGAGAC GGGTATCCCT TCGACGGGAA GGACGGGCTC CTGGCACACG CCTTTCCTCC	600
TGCCCCCGGC ATTCAGGGAG ACGCCCATTT CGACGATGAC GAGTTGTGGT CCCTGGGCAA	660

-continued

GGGCOTCGTG	GTTCCAAC	TG	GGTTCG	AAAA	CGCAGATGGC	GCGGCTGCC	ACTTCCCTT	720
CATCTTCGAG	GGCCGCTCCT	ACTCTGCCTG	CACCACCGAC	GGTCGCTCCG	ACGGGTG	CC	780	
CTGGTGCAOT	ACCACGGCCA	ACTACGACAC	CGACGACCGG	TTTGGCTTCT	CCCCAOCOA		840	
GAGACTCTAC	ACCCGGGACG	GCAATGCTGA	TGGGAAACCC	TGCCAOTTC	CATTCACTT		900	
CCAAGGCCAA	TCCTACTCCG	CCTGCACCAC	GGACGGTCCG	TCCGACGGCT	ACCGCTGOTG		960	
CGCCACCACC	GCCAACTACG	ACCGGGACAA	GCTCTTCGGC	TTCTGCCCGA	CCCGAGCTGA		1020	
CTCGACGOTG	ATGGGGGACA	ACTCGGCGGG	GGAGCTGTGC	GTCTTCCCTT	TCACTTTCCT		1080	
GGGTAAGGAG	TACTCGACCT	GTACCAGCGA	GGGCCGCGGA	GATGGGCGCC	TCTGOTGCGC		1140	
TACCACCTCG	AACTTTGACA	GCGACAAGAA	GTGGGCTTC	TGCCCCGACC	AAGGATACAG		1200	
TTGTTCCTC	GTGGCGGGCG	ATGAGTTCGG	CCACGCGCTG	GGCTTAGATC	ATTCTCTAGT		1260	
CCCGGAGGCG	CTCATGTACC	CTATGTACCG	CTTCACTGAG	GGCCCCCTT	TGCATAAOGA		1320	
CGACGTGAAT	GCCATCCGGC	ACCTCTATGG	TCCTCGCCCT	GAACCTGAAC	CACGGCTCTC		1380	
AACCACCACC	ACACCGCAGC	CCACGGCTCC	CCCGACGGTC	TGCCCCACCG	GACCCCCCAC		1440	
TGTCCACCCC	TCAGAGCGCC	CCACAGCTGG	CCCCACAGGT	CCCCCTCAG	CTGCCCCCAC		1500	
AAGTCCCCCC	ACTGCTGGCC	CTTCTACGGC	CACTACTGTG	CCTTTGAGTC	CGGTGGACGA		1560	
TGCTTGCAAC	GTGAACATCT	TCGACGCCAT	CGCGGAGATT	GGGAACCAAC	TGTATTTGTT		1620	
CAAGGATGGG	AACTACTGGC	GATTCTCTGA	GGGCAAGGGG	AGCCGGCCGC	AGGGCCCCCTT		1680	
CCTTATCGCC	GACAAGTGGC	CCGCGCTGCC	CCGCAAGCTG	GACTCGGTCT	TTGAGGAGCC		1740	
GCTCTCCAAG	AAGCTTTTCT	TCTTCTCTGG	CGGCCAGGTG	TGGGTGTACA	CAGGCGCGTC		1800	
GGTCTGCGG	CCOAGCGTC	TGGACAAGCT	GGGCTGGGA	GCCGACGTGG	CCCAGGTGAC		1860	
CGGGGCCCTC	CGGAGTGGCA	GGGGGAAGAT	GCTGCTGTTT	AGCGGGCGGC	GCCTCTGGAG		1920	
GTTCGACGTG	AAGGCGCAOA	TGGTGGATCC	CCGGAGCGCC	AOCGAGGTGG	ACCGGATGTT		1980	
CCCCGGGGTG	CCTTTGGACA	CGCAGGACGT	CTTCCAGTAC	CGAGAGAAAG	CCTATTTCTG		2040	
CCAGGACCOC	TTCTACTGGC	GCCTGAGTTC	CCGGAGTGAG	TTGAACCAGG	TGGACCAAGT		2100	
GGGCTACGTG	ACCTATGACA	TCCTGCAGTG	CCCTGAGGAC	TAGGCTCTCC	GTCTTGCTTT		2160	
GCAGTGCCAT	GTAAATCCCC	ACTGGGACCA	ACCCTGGGGA	AAGAGCCAGT	TTGCCGGATA		2220	
CAAACTGGTA	TTCTGTTCTG	GAGGAAAGGG	AGGAGTGGAG	GTGGGCTGGG	CCCTCTCTTC		2280	
TCACCTTTOT	TTTTGTTGG	AGTGTTCCTA	ATAAACTTGG	ATTCTCTAAC	CTTT		2334	

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Glu Ala Leu Met Tyr Pro Met Tyr Arg Phe Thr Glu Gly Pro Pro Leu
 1 5 10 15
 His Lys

We claim:

1. An isolated osteoclast-specific or -related DNA sequence, or its complementary sequence, the DNA 65 sequence comprising a nucleic acid sequence selected from the group consisting of:

a) DNA sequences set forth in the group consisting of SEQ ID NOS. 12, 14, 16 and 17, or their complementary strands; and

33

- b) DNA sequences which hybridize under standard conditions to the DNA sequences defined in a).
2. A DNA construct capable of replicating, in a host cell, osteoclast-specific or -related DNA, said construct comprising:
- a) a DNA sequence of claim 1; and
- b) sequences, in addition to said DNA sequence, necessary for transforming or transfecting a host cell, and for replicating, in a host cell, said DNA sequence.
3. A DNA construct capable of replicating and expressing, in a host cell, osteoclast-specific or -related DNA, said construct comprising:

34

- a) a DNA sequence of claim 2; and
- b) sequences, in addition to said DNA sequence, necessary for transforming or transfecting a host cell, and for replicating and expressing, in a host cell, said DNA sequence.
4. A cell stably transformed or transfected with a DNA construct according to claim 3.
5. A cell stably transformed or transfected with a DNA construct according to claim 4.

* * * * *

d

This Page Blank (uspto)



US006340583B1

(12) **United States Patent**
Yan et al.

(10) Patent No.: **US 6,340,583 B1**
(45) Date of Patent: **Jan. 22, 2002**

(54) **ISOLATED HUMAN KINASE PROTEINS,
NUCLEIC ACID MOLECULES ENCODING
HUMAN KINASE PROTEINS, AND USES
THEREOF**

(75) Inventors: **Chunhua Yan, Boyds; Karen A.
Ketchum, Germantown; Valentina Di
Francesco, Rockville; Ellen M.
Beasley, Darnestown, all of MD (US)**

(73) Assignee: **PE Corporation (NY), Norwalk, CT
(US)**

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/813,817**

(22) Filed: **Mar. 22, 2001**

(51) Int. Cl.⁷ **C12N 9/12; C12N 1/20;
C12N 15/00; C12N 5/00; C07H 21/04**

(52) U.S. Cl. **435/194; 435/320.1; 435/252.3;
435/325; 536/23.2**

(58) Field of Search **435/194, 252.3,
435/325, 320.1; 536/23.2**

(56) **References Cited**

PUBLICATIONS

GenEmbl Database, Accession No. D45906, Feb. 1999.*

Sambrook et al., Molecular Cloning Manual, 2nd edition,
Cold Spring Harbor Laboratory Press, 1989.*

* cited by examiner

Primary Examiner—Rebecca E. Prouty

Assistant Examiner—M. Monshipouri

(74) *Attorney, Agent, or Firm*—Celera Genomics; Robert
A. Millman; Justin D. Karjala

(57) **ABSTRACT**

The present invention provides amino acid sequences of
peptides that are encoded by genes within the human
genome, the kinase peptides of the present invention. The
present invention specifically provides isolated peptide and
nucleic acid molecules, methods of identifying orthologs
and paralogs of the kinase peptides, and methods of iden-
tifying modulators of the kinase peptides.

9 Claims, 41 Drawing Sheets

1 CCCAGGGCGC CGTAGGCGGT GCATCCCCTT CGCGCCTGGG GCTGTGGTCT
51 TCCCGCGCCT GAGGCGGCGG CGGCAGGAGC TGAGGGGAGT TGTAGGGAAC
101 TGAGGGGAGC TGCTGTGTCC CCCGCCTCCT CCTCCCCATT TCCGCGCTCC
151 CGGGACCATG TCCGCGCTGG CGGGTGAAGA TGTCTGGAGG TGTCAGGCT
201 GTGGGGACCA CATTGCTCCA AGCCAGATAT GGTACAGGAC TGTCACGAA
251 ACCTGGCAGC GCTCTTGCTT CCGGTGAAAG TGATGCCGAG CCTGGACCAC
301 CCCAATGTGC TCAAGTTCAT TGGTGTGCTG TACAAGGATA AGAAGCTGAA
351 CCTGCTGACA GAGTACATTG AGGGGGGCAC ACTGAAGGAC TTTCTGCGCA
401 GTATGGATCC GTTCCCCTGG CAGCAGAAGG TCAGGTTTGC CAAAGGAATC
451 GCCTCCGAA TGGACAAGAC TGTGGTGGTG GCAGACTTTG GGCTGTCACG
501 GCTCATAGTG GAAGAGAGGA AAAGGGCCCC CATGGAGAAG GCCACCACCA
551 AGAAACGCAC CTTGCGCAAG AACGACCGCA AGAAGCGCTA CACGGTGGTG
601 GGAAACCCCT ACTGGATGGC CCCTGAGATG CTGAACGSA AGAGCTATGA
651 TGAGACGGTG GATATCTTCT CTTTGGGAT CGTTCTCTGT GAGATCATTG
701 GGCAGGTGTA TGCAGATCCT GACTGCCTTC CCCGAACACT GGACTTTGGC
751 CTCAACGTGA AGCTTTTCTG GGAGAAGTTT GTTCCACAG ATTGTCCCCC
801 GGCCTTCTTC CCGCTGGCCG CCATCTGCTG CAGACTGGAG CCTGAGAGCA
851 GACCAGCATT CTCGAAATTG GAGGACTCCT TTGAGGCCCT CTCCCTGTAC
901 CTGGGGGAGC TGGGCATCCC GCTGCCTGCA GAGCTGGAGG AGTTGGACCA
951 CACTGTGAGC ATGCAGTACG GCCTGACCCG GGACTCACCT CCCTAGCCCT
1001 GGGCCAGCCC CCTGCAGGGG GGTGTTCTAC AGCCAGCATT GGGCTTCTGT
1051 GGGCCATTCC TGCTGTGAGC AGGGCCGTCC GGGCTTCTGT TGGATTGGCG
1101 GAATGTTTAG AAGCAGAACA AACCATTCTT ATTACCTCCC CAGGAGGCAA
1151 GTGGGCGCAG CACCAGGGAA ATGTATCTCC ACAGGTTCTG GGGCCTAGTT
1201 ACTGTCTGTA AATCCAATAC TTGCCTGAAA GCTGTGAAGA AGAAAAAAC
1251 CCCTGGCCTT TGGGCCAGGA GGAATCTGTT ACTCGAATCC ACCCAGGAAC
1301 TCCCTGGCAG TGGATTGTGG GAGGCTCTTG CTTACACTAA TCAGCGTGAC
1351 CTGGACCTGC TGGGCAGGAT CCCAGGGTGA ACCTGCCTGT GAACTCTGAA
1401 GTCAC TAGTC CAGCTGGGTG CAGGAGGACT TCAAGTGTGT GGACGAAAGA
1451 AAGACTGATG GCTCAAAGGG TGTGAAAAAG TCAGTGATGC TCCCCCTTTC
1501 TACTCCAGAT CCTGTCTTTC CTGGAGCAAG GTTGAGGGAG TAGGTTTTGA
1551 AGAGTCCCTT AATATGTGGT GGAACAGGCC AGGAGTTAGA GAAAGGGCTG
1601 GCTTCTGTTT ACCTGCTCAC TGGCTCTAGC CAGCCCAGGG ACCACATCAA
1651 TGTGAGAGGA AGCCTCCACC TCATGTTTTT AAACCTTAATA CTGGAGACTG
1701 GCTGAGAACT TACGGACAAC ATCCTTTCTG TCTGAAACAA ACAGTCACAA
1751 GCACAGGAAG AGGCTGGGGG ACTAGAAAGA GGCCCTGCCC TCTAGAAAGC
1801 TCAGATCTTG GCTTCTGTTA CTCATACTCG GGTGGGCTCC TTAGTCAGAT
1851 GCCTAAACA TTTTGCCTAA AGCTCGATGG GTTCTGGAGG ACAGTGTGGC
1901 TTGTCACAGG CCTAGAGTCT GAGGGAGGGG AGTGGGAGTC TCAGCAATCT
1951 CTTGGTCTTG GCTTCATGGC AACCACTGCT CACCCCTCAA CATGCCTGGT
2001 TTAGGCAGCA GCTTGGGCTG GGAAGAGGTG GTGGCAGAGT CTCAAAGCTG
2051 AGATGCTGAG AGAGATAGCT CCCTGAGCTG GGCCATCTGA CTTCTACCTC
2101 CCATGTTTGC TCTCCCAACT CATTAGCTCC TGGGCAGCAT CCTCCTGAGC
2151 CACATGTGCA GGTACTGGAA AACCTCCATC TTGGCTCCCA GAGCTCTAGG
2201 AACTCTTCAT CACAAC TAGA TTTGCCTCTT CTAAGTGTCT ATGAGCTTGC
2251 ACCATATTTA ATAAATTGGG AATGGGTTTG GGGTATTAAA AAAAAAAAAA
2301 AAAAAAAAAA AAAAAAAAAA (SEQ ID NO:1)

FIG. 1A

FEATURES:

5'UTR: 1-228
Start Codon: 229
Stop Codon: 994
3'UTR: 997

Homologous proteins:

Top 10 BLAST Hits

		Score	E
CRA	1000682328847 /altid=gi 8051618 /def=ref NP_057952.1 LIM d...	485	e-136
CRA	18000005015874 /altid=gi 5031869 /def=ref NP_005560.1 LIM ...	485	e-136
CRA	88000001156379 /altid=gi 7434382 /def=pir JC5814 LIM motif...	469	e-131
CRA	88000001156378 /altid=gi 7434381 /def=pir JC5813 LIM motif...	469	e-131
CRA	18000005154371 /altid=gi 7428032 /def=pir JE0240 LIM kinas...	469	e-131
CRA	18000005126937 /altid=gi 6754550 /def=ref NP_034848.1 LIM ...	469	e-131
CRA	18000005127186 /altid=gi 2804562 /def=dbj BAA24491.1 (AB00...	469	e-131
CRA	18000005127185 /altid=gi 2804553 /def=dbj BAA24489.1 (AB00...	469	e-131
CRA	18000005004416 /altid=gi 2143830 /def=pir I78847 LIM motif...	468	e-131
CRA	18000005004415 /altid=gi 1708825 /def=sp P53670 LIK2_RAT LI...	468	e-131

BLAST dbEST hits:

	Score	E
gi 10950740 /dataset=dbest /taxon=96...	1049	0.0
gi 10156485 /dataset=dbest /taxon=96...	975	0.0
gi 5421647 /dataset=dbest /taxon=9606 ...	952	0.0
gi 10895718 /dataset=dbest /taxon=96...	757	0.0
gi 13043102 /dataset=dbest /taxon=960...	714	0.0
gi 519615 /dataset=dbest /taxon=9606 /...	531	e-149
gi 11002869 /dataset=dbest /taxon=96...	511	e-143

EXPRESSION INFORMATION FOR MODULATORY USE:

library source:

From BLAST dbEST hits:

gi|10950740 teratocarcinoma
gi|10156485 ovary
gi|5421647 testis
gi|10895718 nervous normal
gi|13043102 bladder
gi|519615 infant brain
gi|11002869 thyroid gland

From tissue screening panels:

Fetal whole brain

FIG.1B

1 MVQDCQRNLA RLLLPVKVMR SLDHPNVLFK IGVLYKDKKL NLLTEYIEGG
51 TLKDFLRSMDFPWPQKQVRF AKGIASGMDK TVVVAADFGLS RLIVEERKRA
101 PMEKATTKKR TLRKNDRKKR YTVVGPNPYWM APEMLNGKSY DETVDIFSFG
151 IVLCEIIGQV YADPDCLPRT LDFGLNVKLF WEKFPVPTDCP PAFFPLAIC
201 CRLEPESRPA FSKLEDSFEA LSLYLGEGLI PLPAELEELD HTVSMQYGLT
251 RDSPP (SEQ ID NO:2)

FEATURES:

Functional domains and key regions:

[1] PDOC00004 PS00004 CAMP_PHOSPHO_SITE

cAMP- and cGMP-dependent protein kinase phosphorylation site

Number of matches: 2

1 108-111 KKRT

2 119-122 KRYT

[2] PDOC00005 PS00005 PKC_PHOSPHO_SITE
Protein kinase C phosphorylation site

Number of matches: 4

1 51-53 TLK

2 106-108 TTK

3 107-109 TKK

4 111-113 TLR

[3] PDOC00006 PS00006 CK2_PHOSPHO_SITE
Casein kinase II phosphorylation site

Number of matches: 4

1 51-54 TLKD

2 76-79 SGMD

3 139-142 SYDE

4 212-215 SKLE

[4] PDOC00008 PS00008 MYRISTYL
N-myristoylation site

Number of matches: 4

1 73-78 GIASGM

FIG.2A

2 77-82 GMDKTV

3 150-155 GIVLCE

4 158-163 GQVYAD

Membrane spanning structure and domains:

Helix	Begin	End	Score	Certainty
1	142	162	0.872	Putative
2	184	204	0.652	Putative

BLAST Alignment to Top Hit:

>CRA|1000682328847 /altid=gi|8051618 /def=ref|NP_057952.1| LIM
domain kinase 2 isoform 2b [Homo sapiens] /org=Homo
sapiens /taxon=9606 /dataset=nraa /length=617
Length = 617

Score = 485 bits (1235). Expect = e-136
Identities = 241/265 (90%). Positives = 241/265 (90%). Gaps = 22/265 (8%)

Query: 13 LLPVKVMRSLDHPNVLFKFIGVLYKDKKLNLLTEYIEGGTLKDFLRSMDFPWPQQKVRFAK 72
L VKVMRSLDHPNVLFKFIGVLYKDKKLNLLTEYIEGGTLKDFLRSMDFPWPQQKVRFAK
Sbjct: 353 LTEVKVMRSLDHPNVLFKFIGVLYKDKKLNLLTEYIEGGTLKDFLRSMDFPWPQQKVRFAK 412

Query: 73 GIASGM-----DKTVVADFGLSRLIVEERKRAPMEKATTKKR 110
GIASGM DKTVVADFGLSRLIVEERKRAPMEKATTKKR
Sbjct: 413 GIASGMAYLHSMCIHRDLNSHNCILDKTVVADFGLSRLIVEERKRAPMEKATTKKR 472

Query: 111 TLRKNDRKKRYTVVGNPYWMAPEMLNGKSYDETVDIFSFGIVLCEIIGQVYADPDCLPRT 170
TLRKNDRKKRYTVVGNPYWMAPEMLNGKSYDETVDIFSFGIVLCEIIGQVYADPDCLPRT
Sbjct: 473 TLRKNDRKKRYTVVGNPYWMAPEMLNGKSYDETVDIFSFGIVLCEIIGQVYADPDCLPRT 532

Query: 171 LDFGLNVKLFWEKFVPTDCPPAFFPLAAICCRLEPESRPAFSKLEDSFEALSPLYLGELGI 230
LDFGLNVKLFWEKFVPTDCPPAFFPLAAICCRLEPESRPAFSKLEDSFEALSPLYLGELGI
Sbjct: 533 LDFGLNVKLFWEKFVPTDCPPAFFPLAAICCRLEPESRPAFSKLEDSFEALSPLYLGELGI 592

Query: 231 PLPAELEELDHTVSMQYGLTRDSPP 255
PLPAELEELDHTVSMQYGLTRDSPP
Sbjct: 593 PLPAELEELDHTVSMQYGLTRDSPP 617 (SEQ ID NO:4)

Hmmer search results (Pfam):

Model	Description	Score	E-value	N
PF00069	Eukaryotic protein kinase domain	100.1	1.1e-26	2
CE00031	CE00031 VEGFR	4.9	0.14	1
CE00204	CE00204 FIBROBLAST_GROWTH_RECEPTOR	4.7	1	1
CE00359	E00359 bone morphogenetic protein_receptor	1.8	7.9	1
CE00022	CE00022 MAGUK_subfamily_d	1.5	2.5	1
CE00287	CE00287 PTK_Eph_orphan_receptor	-48.4	3.8e-05	1
CE00292	CE00292 PTK_membrane_span	-61.8	2.1e-05	1

FIG.2B

CE00291	CE00291 PTK fgf_receptor	-113.0	0.027	1
CE00286	E00286 PTK EGF_receptor	-125.1	0.0021	1
CE00290	CE00290 PTK Trk_family	-151.3	6.5e-05	1
CE00288	CE00288 PTK_Insulin_receptor	-210.4	0.014	1

Parsed for domains:

Model	Domain	seq-f	seq-t	hmm-f	hmm-t	score	E-value
PF00069	1/2	16	79 ..	41	105 ..	52.1	2.3e-13
CE00022	1/1	124	153 ..	187	216 ..	1.5	2.5
PF00069	2/2	81	156 ..	129	182 ..	48.0	3.1e-12
CE00031	1/1	129	156 ..	114	1141 ..	4.9	0.14
CE00204	1/1	129	156 ..	705	732 ..	4.7	1
CE00359	1/1	79	157 ..	287	356 ..	1.8	7.9
CE00290	1/1	9	218 ..	1	282 []	-151.3	6.5e-05
CE00287	1/1	1	218 [.	1	260 []	-48.4	3.8e-05
CE00291	1/1	1	218 [.	1	285 []	-113.0	0.027
CE00292	1/1	1	218 [.	1	288 []	-61.8	2.1e-05
CE00288	1/1	1	218 [.	1	269 []	-210.4	0.014
CE00286	1/1	6	218 ..	1	263 []	-125.1	0.0021

FIG.2C

1 TCATCCTTGC GCAGGGGCCA TGCTAACCTT CTGTGTCTCA GTCCAATTTT
51 AATGTATGTG CTGCTGAAGC GAGAGTACCA GAGGTTTTTT TGATGGCAGT
101 GACTTGAAC TATTTAAAAG ATAAGGAGGA GCCAGTGAGG GAGAGGGGTG
151 CTGTAAAGAT AACTAAAAGT GCACTTCTTC TAAGAAGTAA GATGGAATGG
201 GATCCAGAAC AGGGGTGTCA TACCGAGTAG CCCAGCCTTT GTTCCGTGGA
251 CACTGGGGAG TCTAACCAG AGCTGAGATA GCTTGCAGTG TGGATGAGCC
301 AGCTGAGTAC AGCAGATAGG GAAAAGAAGC CAAAAATCTG AAGTAGGGCT
351 GGGGTGAAGG ACAGGGAAGG GCTAGAGAGA CATTTGGAAA GTGAAACCAG
401 GTGGATATGA GAGGAGAGAG TAGAGGGTCT TGATTTCGGG TCTTTCATGC
451 TTAACCCAAA GCAGGTACTA AAGTATGTGT TGATTGAATG TCTTTGGGTT
501 TCTCAAGACT GGAGAAAGCA GGGCAAGCTC TGGAGGGTAT GGCAATAACA
551 AGTTATCTTG AATATCCTCA TGGTGGAAAG TCCTGATCCT GTTTGAATTT
601 TGGAAATAGA AATCATTAG AGCCAAGAGA TTGAATTGTT GAGTAAGTGG
651 GTGGTCAGGT TACAGACTTA ATTTTGGGTT AAAAAGTAAA AACAAGAAAC
701 AAGGTGTGGC TCTAAAATAA TGAGATGTGC TGGGGGTGGG GCATGGCAGC
751 TCATAAACTG ACCCTGAAAG CTCTTACATG TAAGAGTTCC AAAAATATTT
801 CCAAACTTG GAAGATTCAT TTGGATGTTT GTGTTTCTTA AAATCTCTCA
851 CTAATTCATT GTCTTGTTCA CTGTCCGTAA CCAACCTGG GATTGGTTTG
901 AGTGAGTCTC TCAGACTTTC TGCCTTGGAG TTTGTGAGAG AGATGGCATA
951 CTCTGTGACC ACTGTCACCC TAAAACCAA AAGGCCCTC TTGACAAGGA
1001 GTCTGAGGAT TTTAGACCCA GGAAGAATGA GTGATGGGCA TATATATATC
1051 CTATTACTGA GGCATGAGAA GAGTGAATG GGTGGGTTGA GGTGGTGTTC
1101 TAAGGCCTCT TGCCAGCTTG TTTAACTCTT CTCTGGGGAA CGAGGGGGAC
1151 AACTGTGTAC ATTGGCTGCT CCAGAATGAT GTTGAGCAAT CTTGAAGTGC
1201 CAGGAGCTGT GCTTTGTCTA TTCATGGCCC CTGTGCCTGT GAAACAGGGT
1251 TCGGTGACTG TCACTGTGCC TGTGGCAGTC TGAGTTACC CAGAGAGAAC
1301 AAAGCTGCAT ACACAGAGCG CACAAGGGAG TCTTGTAACA ACCTTGTCCT
1351 GCTTTCTAGG GCTGAGTCAG GTACCACAGC TTGATCTCAG CTGTCCTCTT
1401 TATTTCAAGA AGTTGACATC TGAGCCATAC CAGGAGTATT GTATTTTGT
1451 TGAGGCCTCT CTTTTTGGAG GAACATGGAC CGACTCTGTG CTTTTGTCTA
1501 TGCTGGTCTC TGAGCTCACA CAACCCTTCA CCTCCTTTC TCAGCCAGTG
1551 ATAGGTAAGT CTTCCCTATC TTGCAAGGCT CAGCTCAAGT GTCAGCTTCC
1601 TCTACAAAGA CTTTCCTGGT TCCCCTCATT GGAGTGAACA AGAGTTGACA
1651 TGGTAGAATG GAAAGAGCAG AAGCTTTAGA ATGAGCCAGA CCTGAGTATG
1701 AATGCTAGAT CCACCACTTA GCTAGTCAAC CCTGCCCCCT GCCTCAAGTT
1751 TTAATTTTCC TATCCATTAA GTGAATATAA TAATACCTGT GTCACAGGAT
1801 TATTTTGAGA ATTAATGAG ATTAGGTCTA TGAAAGCACC TAGCAGAGTT
1851 CTTGGCATAT AGGAGGCATT CATTAAATAT TTGTTCTTCC CTTTTATAC
1901 CCATTACTTT TCTTTTCTG AACTAAAATA AACTTTGGTT CTATCTCTGA
1951 AATAACATCC AAGTGAAAAA TCAACAACAT GAAAGAGCAG TTCTTTTCCA
2001 GTGGATTTGC TTCTTAAGGA GCAGAGATTA TGTAATCTAA CAGCCTCCAA
2051 CATACAAAGA GCTTTGTATC TAGAACAGGG GTCCCCAGCC CCTGGACCGC
2101 CAACTGGTAC GGGTCTGTAG CCTGTTAGGA ACCAGGCTGC ACAGCAGGAG
2151 GTGAGCGGCG GGCCAGTGAG CATTGCTGCC TGAGCTCTGC CTCCTGTCAG
2201 ATCAGTGGTG GCATTAGATT CTCATAGGAG TGTGAACCCT ATTGTGAAGT
2251 GCACATGCAA GGGATCTGGG TTGCATGCTC CTTATGAGAA TCTCACTAAT
2301 GGCTGATGAT CTGAGTTGGA ACAGTTTGAT ACCAAAACCA TCCCCCGCC
2351 CCCCACCCC CAGCCTAGGG TCCGTGGAAA AATTGGCCCC TGGTGCCAAA
2401 AAGGTTGAGG ACTGCTGATC TAGAGGACCA ATTTATTCAA TGTGTTGGA
2451 GTAAATGAGC TCTTGGATTA GGTGATGGAA AAATCTGAAA AAACAGGGCT

FIG. 3-1

2501 TTTGAGGAAT AGGAAAAGGC AGTAACATGT TTAACCCAGA GAGAAGTTTC
2551 TGGCTGTTGG CTGGGAATAG TCATAGGAAG GGCTGACACT GAAAAGAAGG
2601 AGATTGTGTT CGTTTCTTCT TCTCAGAGCT ATAAGCAAAG GCTGAAAGTT
2651 CTAGAAAAAG GCAAGTTTTG TTTCAGTAGA AAAAAGGATA ATCAGAACCA
2701 TTTTGTAGAA ATGGAATGAG ACTACTTTTG AGGCCATGAG TTCCTTGTC
2751 CTGGAGAGAT GAGCAGAGGT TGGACAAGTG CTTACCAGAG ATCTTGTTGA
2801 GGCAGAAACT GTGCATCTAG CAGAGCATTG GCCTAACCCT TTCAAATGAG
2851 ATGCTGTAA CTCAGTCTTA TTCTACATGG TAGGAATCCT GTCCCTTTC
2901 CTCCTGCTAC TTTGGGCCTC TCAACCTCTT GGTTTTGTGT GCAGGTGAAG
2951 ATGTCTGGAG GTGTCCAGGC TGTGGGGACC ACATTGCTCC AAGCCAGATA
3001 TGGTACAGGA CTGTCAACGA AACCTGGCAC GGCTCTTGCT TCCGGTAGGT
3051 GGGCCTATCC TCCCATCTTT ACCAGTGATC TATGGGCCAA GCACTATTTT
3101 ATGTTCTGAT GGAAAACACA GAAACAAGCT TCTGAGTTGA GAATTTCAAT
3151 CTTAGGGTGG GGAAAGGAAT GTACCAAGGA AGAGCTCATG ACCAAACCTC
3201 AAGTGTGGCC CCCCTGAACC CAGGTAAAT TGGAGAGCC ATAAATGGGC
3251 CAGCTGGAGG CAGGGTGGGG GGATGAGAGG AGCCCTTTCC AGGGTTGTCC
3301 CATATCCCTC ACTTTATGGG TGAGGAACT GAGGCCAGG AAGAGTGACT
3351 TTCCTGTGGC TGCACTACAG ATTATGCAGG TACTTCAAGA GTTGTGTTGA
3401 TTCTTATTTT ATTTTATTTT ATTTTATTTT ATTTTATTTT ATTTTATGAG
3451 AGGGATTCTT GCTGTTGCCC AGGCTGGAGT GCAGTGGTGC AATCTCGGCT
3501 CACTGCAATC TCTGCCTGCT GGGTTCAAGT GATTTTCTG CCTTAGCTTC
3551 CTGAGTAGCT GAGATGACAG GCACCTGCCA CCATGCGCAG CTAATTTTTG
3601 TATTTTAGTG GAGACGGGGG TTTCAACATG TTGGTCAGGC TGGTCTTGAA
3651 CTCCTGACCT CAAATGATGC ACCCACCTCG ACCTCCCAA GTGCTGGAAT
3701 TACAGGCGTG AACCCTGTG CCCAGCCAAG AGTTGTTTTT AGTGTGGTTG
3751 GCAGAGCCAG CTCTTCCTTC ACCACAGGAT GCCTCCCTAG GTTCTACTT
3801 TTTGTTACTA GCTTTTATTA TAGCTATATT ATTATTATTA TTATTATTAT
3851 TATTATTATT ATTATTGAGA CAGAGTCTCG CTCTGTCGCC CAGGCTGGTG
3901 TACAGTGGTG CGATCCCGGG CTAAGTCAA CCTCTGCCTC CCGAGTTCAA
3951 GCAGTTCTCC TGCTCAGCC CCCCAGTAG GTGGGACTAC AGGCGCTGC
4001 CACCACACCC GGCTAATTTT TGTATTTTGA GTAGAGACGG GGTTCACCT
4051 TGTTGACCAG GCTGGTCTGG AGCTCCTGAC CTCAGGTAAG TGCTAGAATC
4101 ACAGGCGTGA ACCACTGCGC CCAGCCAAGA GTTGTTTTTA GTGTGGTTGG
4151 CAGAGCCAGC TCTTCCTCAC CACAGGTTGC CTCCCTAGGT TCCTACTTTT
4201 TGTTACTAGC TTTATTATAG CTACATTATT ATTATTATTG TTATTATTAT
4251 TGAGACAGAG TCTCGCTCTG TCGCCAGGC TGGTGTACAG TGATGTGATC
4301 TTGGCTCACT GCAACCTCTG CCCCCGAGT TCAAGCAATT CTCCTGCTTC
4351 AGCCCCCTA GTAGGTGGGA CTCCAGGCAC CTGCCACCAC GCCCAGCTAA
4401 TTTTGTATT TTTAGTAGAG GCGGGGTTTC ACCTTGTTGG CCAGGCTGGT
4451 CTCAAACCTC TGACCTCAGG TGATCCGCCT GCCTCGGCT CCCAAATGT
4501 TGGGATTACA GGCATGAGCC ACCGCGCCT GCCTATAGCT ACATTATTTT
4551 TGTAGGCAGC TCAGTTTCTT AAAAATTATA CAGACTCAA ATCAGATTG
4601 TTCCTGCTGT CTGAGGCTCA GTTCTTCAT CTGGAAAATG GATGGTAATA
4651 ATCTTGTTGA GATTGAATGA AATAATATAT GCAGTGTATC CAGTACATGG
4701 TAGACACCCA GTGAATGGT ATTCTTCCT CCCATCGGAT TGGAAATCTC
4751 AAGGGTGGGA ACTTGTCTTT ATATTCTTCA CAACGTAAAA TAGTTGAAAT
4801 TTGTTGGTGG AAAGAAGAGC AGTCCACTCC AGAGGCTGGA TGGGCATGCC
4851 TGGCCCCCAA GGTCTGAAGT GGTAGGGCTG TGCCTATATC CTGAGAAATGA
4901 GATAGACTAG GCAGGCACCT TGTGCTGTAG ATTCCAGCTC CTGCACATAG
4951 CTCTGTTGT AAAACATCCC TGTGCTTATA CCAAGTAATT GAGTTGACCT

FIG.3-2

5001 TTAAACACTT GCCTCTTCCC TGGGAACCAT ATAGGGGATT GGCCTGGAGA
5051 CGTCTGGCCT CTGGAAGAGT TGGAAAGCAG CCATCATTAT TATCCTTTCC
5101 TTTCAGCTAT AACTCAGAGC TCTCAAGTCT TTTCTGTGGA TCTTATTGCC
5151 TTGGTTCTTG CCCCTTTTAC TCCCAGGGAA GTTGATTCTG TCTTTTCTGT
5201 TCCATTTAGT ATGACAGGAG CAGAGAATGT CAGAGCTGTA AGGGACCTTA
5251 TAGTTAAAGC CTTTGGCTGG TCCTTTCATT TTATAGCTGG GACTAATAAG
5301 TAACGTCAAA ACCCAATGAG TTCACAGATT GGGTCTCGCC TTGGCATGTA
5351 ACCCATATGT TCATATTCTT GCTGTTTTCC TATGTGTATG AATATTTTCT
5401 ATCCAAAATA AGCAGGACAG GGTAGAGCAA GTTAATCTTT GGAATTTCTG
5451 GATTCTCTTA GAGCTAAAAA ACTTCAGAAC TAGAAGAAAC CACCCACTAT
5501 ATGGTATAAC CCATTCATAT CACAGATGAG GCCTGAAACC AAAAAGACTT
5551 GCTCAGGCCA TGGATGACAA GAGCTGGCCC TAGCACTGAA CTCTTGGGTC
5601 ATTTGTAGGT CTAGTCAGAT GCTAGCTTGT TAGCTCTGTG CGTGCGTGTG
5651 TGTGTGTGTG TGTGTGTGTG TGTGTGAGAT AGAGACAGAA AGATAACATA
5701 TGTACACAAA TACATAAAGA GGAAGTAGAC ACGTTAGCAT GGTAGATAAG
5751 AGTACAGGCA GGCCAGGCGT GGTGGCTCAC GCCTGTAATC CCAGCACTTT
5801 GGGAGGCCAA GGCAGGTGGA TCACCTGAGG TCAGGAATTC GAGACCAGCC
5851 TGACCAACAT GGTGAAACCC CATCTCTACT AAATACAGAA AAAAATTAGC
5901 TTGGCATGGT GGCACATGCC TGTAATCCCA GCTACTTGGG AAGCTGAAGC
5951 AGGAGAATCG CTTGAATCCG GGAAGCAGAA GTTGCAGTGA GCCGAGATTG
6001 TGCCATTACA GTCTAGCCTG GGCAACAAGA GGGAACTCC ATCGCAAAAA
6051 AACCAACCACC ACCAAGAGTA CAGGCTATGG AATGAGACTA TGGTTTTAAA
6101 TCCTGGCTTT GCAATTTATT AACTAGCCTT AAGTGACTTC CCTGAGCTTC
6151 AGGCACCAAT CTGTAAAATG AGGATAAGAA TATTACTCAT GCCACATGGT
6201 TGTTAGGGAG GATTAAATGT GATAACCTAT ATAAAGTGGC TAGCATAGCA
6251 TCTGACATAT AGAAAACTCT TAATAGGGCC GGACGTGGTG GCTTATGCCT
6301 GTAATCCTAG CACTCTGGGA GGCCGAGGCA GAAGGATCGC TTGAGCCCAT
6351 GAGCCCAGGA GTTTGAGACC AGCCTGGCCA ACATGGCAAA ACTCCACCTC
6401 TACAAAAAAT AAAAAATAT TAGCCAGGCG TGATGGCACA CACCTGTAGT
6451 CCCAGCTACT TGGGAAGCTG AGGAGCGATG ATTACCTGAG CCCAGGGATA
6501 TCAAGGCTGT AGTGAGCTGT GATCATGCCA CTGTACTCCA TCCAGCTGGG
6551 GGACAGAGTG AAACCCCTGT CTCAAAAA AAAAAATGAA AAAAAAACC
6601 CTTAATAATC AGTAACTGTC ACTTTATATT ATGTTGTGAG TGTGTGTCTA
6651 TATACACCTA TATGTATACA TTTCTCTTAT TACACATTCA TTGGTGATCT
6701 GATGTGGAGC CCCAGGGATT AAGGGCAACT TTGAACTACC CTGACACAAT
6751 CAAGCCAAAT ATCATTCCCG TGGAGGAAGT AGAGTATCTA GGTTCTGTCT
6801 CCTAGTTGCA GCTTTACCTT GAGGACAGAG ACTCTAATCC AGCTGTGCTG
6851 AAGGAGCACA TCTCCTGACT TCTGAGCTTT CCCCTGGTAA ATTCAAACCTG
6901 GATGTCACGG CGCCCTCAGA TAGAGCCTGG TAATTTGCCC TGGGGAGAGT
6951 GACTGTCTTT TGGATCTAAT TTGACTTTTG CCCAGTTGG AGGAAAACTC
7001 TCAGGGCTAG GAAGGATTGT ATTTGTCTGA CCCAGAGAT AACCTGGGTT
7051 TTGAGGAACA TGGGGCATCA ACCTGAATGG TCTTGTAAGA TCTCTCCAC
7101 GCCAGCTTGC CAGTGTCTTCT CTGATGAATT TAGAGTACCT GAGTAGTGCA
7151 GGCCTGCTGG GAGGAGGACT CTCCTCTGT GCTACTCAGA GAAATTCAAT
7201 CTTCAAGGCC CCCTTCCAGC CTTGCTCTTA CCCAGCTGGG CTACAGTTAC
7251 AATAAAGGAA ATGACTTTTC TTCTCCCTT CCCCAGTAC CTTTGTTTTC
7301 CTAGTCACAG GGTGGGGCTG GATATTGAAT GGAGAAATTG CTGGGGTCCA
7351 TCCTAAACTC CTCCCTCAT CTCTCCCTTA CATTACCCCA TTCTTCTGTC
7401 TGCAGCCACA TCCATAATCC TGCTCTGTT AGCCTTCCGA CAGACCCCTA
7451 GGTGCCCAGG ACAACAGGAA GCTACTTAA GCTGGAACCT CAGACTGTGC

FIG. 3-3

7501 AATGGAGGCC AGTGACAAAA CTGAAAGTAG CTCTGTCACT AATTGTGCTG
7551 GTGCGATTAG GCAGCTGGCC AGAATCTTTT GGATCTCCTG GACATATGGC
7601 TGA CTAGTCC TCCCAAGCCT TCCCAACAGG CCTCTTTTTT TTCTTTTTT
7651 TCTTTTCTTT TTTTCTTTT TTTCTTTCTT TCTTTTTTTT TTTTTTTTAG
7701 GCTAGTGAAG TGA AATTGTG GGAGTGGAAG AGGAACAAAG AAATCGGTAA
7751 CTGGTAGTGA TCAATTACTT GTAAACACTA TTGTACTTGG ACCAGCCCAG
7801 TAGGCCTTTT TTA AACTCT GAGTTACCTC TCTTTCCTTT CCTTGAGCAG
7851 TGCCATTAAT TCTGTATCTG GGGCAATCCT TTCTGATGTT CTCTGGACCT
7901 GGCTCTCTCT CCTTAGGAGA GGCCAGGAGA GTAGCCAGAG AGCATGTCTAT
7951 TTGTAGCTGA GGT TAAAGTG TGGAGCTATC AATGGTGACC TGGCCTCTTG
8001 GCATGTTAGC AAGCCAGAGG ACCTTGACAA CTTTTTTGAT GATTGTCCGT
8051 TCACCCTGAT CAAAGGTGTT TGGCTTAGGA GGAGGGAAGA AAAGCTACCC
8101 CTATTAGTCT TGATGGCCCC AGCGTGGGTC TCTATTGCTT GACCTGGTTC
8151 CTAGCAGCAT TATCAGAAGG AAAATCCACC GCTCTTAAGG CTCCTGGGAA
8201 CTTTCAGGAC TTCTTTCTC AGGATTGCAA ACATAAGACT ATTTGAGCTT
8251 TCACTTTTGA AAAGCGGTTA CTAATACCTA TACTCTGGGA AAGGGCTAAT
8301 GCAGATAGAA GACTGTGGTC ACTGCATCAG GCAACAGACC ATTTCCGCTA
8351 AATTTAGTGA CTCCAGGAAG GCCAGTGAAG AAATAACACA CGTAGCAACC
8401 AGAGACTGTG TTGTAATATG TTGGCTGACA GCAGGGTACT TTCTGTGATG
8451 CTGAAAGCCA CATTCATTTT CTCTCCCCTC ATCCCATCT AAGCAAGCCT
8501 GGTAGAATCA TAATTACAGT AATAGGTACC ACTTATTGAG TACTCTGTGC
8551 CAGACACCCT CCTGAGCATA CGACATGCAT AGCACATTTA ATCCTTACAA
8601 TGA CTTAATA AAATGTAGTA CTAGTCTTAC CTACTTCGAG AATAGGGAAA
8651 TGGAGGTTAC TTGTTTAAAG TCACAGAGCT AATAGGTAGC ATAGCTGAGA
8701 TTTGAACTCA GGCATTCTTA CTCCTTGCTT GCAAGAGTCT CTTGGCATTCT
8751 TTGAATGCAA GCATATTTCT TAACCTCACT GAGGCTCAGT TTCCTCTTAT
8801 ATAATATGGG GTAAAGAGCC CTCACCCTGC CTGCCACACA CTGGTAGTGT
8851 CAGATAACAT TGAAGGGTGT TAGTTTAAAG GCTTCATGGA CTCTATAATG
8901 TCAACAAAAG TGCTGTTAAC TTTCTTCTGG GTCTCAGGCT CCTGATGTAG
8951 AGTCAGTGGA GCAACCCTGC CATCTGCTGT TATGCTGTTG ATGTTGCTGC
9001 CACACTTACT AACCTAAACC TTTGATTCTG GCTGTGGCCT TCTCCAGAAG
9051 GTGTTTACTC ATTTGTCCAG TTTATCTTTT AGGAAACAGC CAGCCCCTAG
9101 ATCATTAAAG CTGGCTATTG GACAGGGGGC TGGGGCCTGC CTGACAGAGG
9151 AAGGAAGGGC AGACATCTGG TTCTTCTCTT GCCCCTACAA GAGACTCCAG
9201 CCTGACCACA GAGTGGTACT CCTAGGATGT AGCAGCAGCA TATGAGCTTG
9251 AATGTGCCTT AATCCTGCTC TTTACTTTGA GAAGAGAGAA CTAAGGACCC
9301 ACAGATGTTT CACAGCTTCT ATAGGAGGCA GAGGTAGAAA AATGGAGAGA
9351 GATGAGGCCA GAGATAGATA ACTGATATTA ATTAACGTT GTATTAAGAA
9401 CCTCACTTAG ATTATCTGAT TCAATCTTCA TAATAACCCT GCAACCCCCA
9451 CCTTTTTTTG AGAACAGGGT CTTGCTCTGT TGTCCAGGCT ACAGTGCAT
9501 GGTACAATCA TAGTTCCTG CAGTGTCAAC CTCCTGAGCT CAAGCAATCC
9551 TCCCACCTCA GCCTTGCAAG CAGCTTGGAC TACAGGCGTG CCACCACACC
9601 TTGCCATTTT TTTTATTTT AAGTAGAAAC AAGGTCTTAT TAATACTATG
9651 TTGCCAGGC TGGTCTTGAA CTCCAGCGAT CCTCCTGCCC CAGCCTCCCA
9701 AAGTGCTTGG GATTACGGAA GTAAGCCACT GTGCCTGGCC AGTGCAACCC
9751 CCATTTTATA CTA AACAGG AAGGCCAGA AAGGTTTGA GTA ACTTGTG
9801 CAGGGTCACA CAGATGATAT TTGAACTCAG GTCTCCCTGG CTCCAAGAG
9851 AGTCTGCTTT CCACTAGGAC TCCAGGAGA AAAAAAAAAA AAAAAACAGT
9901 AGACTTGGAG ACAGAAAATC TGATTTGAGT CTTAGTTGAG CTAGGCTAAC
9951 TGTGTAAC TG GCAAGTT CCTTAGCCCC TGTGAGCCTC AGTTTCTTAT

FIG.3-4

10001 CTGTAAATG TCATAAAGA AATCCATCTC ATGGAGTAGT TGTGATGATC
10051 AAGGACTCTG AAAACATTAG AATGGTTTAA TGTGAAGGAT TAGCAGCAGC
10101 ACATGGCAAC ATTGTGCATC TTATATTAAC TATCCAAATA TATCAAGCGT
10151 CATTGCTAT ATATAAAGT CATCAAATTA GGCAGTGTGG GGGATACGGA
10201 GTTGGCATAC TAGCCTGGCC TCTTAATTAA TTCATTAATT AGCTTATTTA
10251 TTTTGTAGAT AGGTCTTGCT CTATTGCCCA GGCTGGAGTG CAGTGGCATG
10301 ATGATAGCTT ACTATAGCCT CAATCTCCCA GGCTTAAACA ATCCTCCTGA
10351 GTAGCTGGGA CTACAGGCAC AACTACCAT GCCAGCTAA TTTTTTTTA
10401 ATTTTTGTGA GAGACAGGGT CTTGCTCTGT TGCCAGGCT GGTCTCAAAC
10451 TCCTGGGCTC GAGATCCTCC CACCTGGGCC TCACAAAGTG TTGGGATTAC
10501 AGGTATGAGC CACGGCACCT GGCCTGGTCT CTAACTGGT TCCCTAAGAC
10551 AGCTGGAAT AGAGAATGTC ATGGAGCATT CTAACCATG GGCTCCAGCC
10601 TGGCTTTCAT TCTGTTTCTC CCCTGAAACA ACATTCTTT AGTAATATTC
10651 CGAATAACAG CTTATCAGT CTGTCTACCG ACCACTCTTC AGGCTTCATC
10701 TTATATGACC TCCCAAATG CACTAAGGGT TGTATTAGAG AAAAGTGGAT
10751 AAAGTTCGGA GTCAGGCTGC TTGAGCTTAA ATGCCAGCTT CACTTACCAG
10801 CCACCTGACC ATGAGTCAGC TGCTTAACCA TTCTTTGCCA CAGTTTCCTT
10851 GTCTATGAAA AGGGAAATGG CTCCACCTC AAAAAGTTGT TAACATTA
10901 TTCAATCATG TATTCAAAGT CCGAGCAGA ATGTCTGGCC ATGACTGGGA
10951 CTTAACAGAT GTTAGCATTT ATTATTAGTA TCTGTCAGTC TTGAAATGTT
11001 CTCTCCCTT GGCTTTCATG ACATTCCACA CTCTCCTGGT TTTCTCTTAC
11051 CTCTCTGGTA ATACCTGTTT GCTTATCCTT CTTTGTCCAG CTCTGGGATG
11101 TTACCATTC TACAGCGTG CTGTTTCTC CTTAGGCAGT CTTACACACA
11151 CTCATGACTT CTTCCATTG TCCTCCACAC ACTGATGACC CTAAATCAG
11201 TATCTCCAGC CTAAACCTTT CCACTGAGTT CTAGACCCAT ATGTTGTACT
11251 ATCAACCTGG CTTGTCCATT TGAATGTCTT CCAGGCACTT CAGACTCTCT
11301 TCTCTAGACT TTGCTGGACT TTCACTCTC CCCCTAAAC TGGCTCCTCT
11351 TCCACTGAAA CATGTATGTC ATTGAGAGGC ACCACCATCC ACCCAGTGCC
11401 TAAGCCAGAA ACCTAGGAAT CTTGATACC GTTCTCTCT CATCCTGCAT
11451 ATCCAAGCCT ATCAGTTTTA TCTCTAAATT ATATTTTGGT AGGTTTACTT
11501 CTTTCTTTT CTCCCACCAC CACCCTGCTC CAAGCTACCA TCATCTCACC
11551 TGGATGTCTG CAATAGCCTC ATCTCCACA GCCACTCTGC ACCCCCTAAT
11601 CTGTTCTCTA TAGAGCAGTT GGAAGGAGTG ATTTTGTGT TTTGTTTGT
11651 TTTGTTTTAG ACAGAGTCTC ACTCTGTTCC CCAAGGCTGG AGTGCAGTGG
11701 CACAATTTTC GCTCACTGCA ACTTCTGCCT CCCGGGTTTA AGCAATTCTC
11751 CTGCCTCAGC CTCCCAAGTA GCTGGGATTA AGGCACCGGC CCCCATACCC
11801 AGCTAATTTT TATATTTTGA GTAGAGATGG GGTTTTGCCA TGTGTTGCAA
11851 GCTAGTCTCG AACTCCTGAC CTCAAGTGAT CCACCTGCCT CGGCCTCCCA
11901 AAGTGCTGGG ATTACAGGTG TGAGCCACTG CACCTGGCTG GAAGGAGTGA
11951 TCTTAAAAA AAAAAAACA AAAAAAAT TGACTGTGTC ACTCTGTGTT
12001 GTCTCTCCTA CTTGTATAC TTCCACAAC TCCAGTGTT CTTGGATAAA
12051 GACCAAAATC CTTAATTGG CCAGGCGCGG TGGCTCACAC CTATCATCTC
12101 AGCACTTTGG GAGGCCGAGG CAGGCAGATC ATGAAGTCAA GAGATTGAGA
12151 CCATCCTGGC CAACATGGTG AAACCCCATC TCTACTAAAA ATACAAAAAT
12201 TAGCTGGTCG TGGTGGCGTG TGCTGTAGT CCCAGCTACT TGGGAGGCTG
12251 AGGCAGGAGA ATCACTTGAA CCTGGGAGGC AGAGGTTGCA GTGAGCCAG
12301 ATCACGCCAC TGCACTCCAG CCTGGTGACA GAGTAAGACT CCATCTCAAA
12351 AAAAAAATA AAAAAAATA TTCCTTAATT TGGCTACAG TAGAGCCCTC
12401 CGTAATGTGG CCTCTCTCCA CATCTCCACA ACCTCCTGCT CCCTGCACTT
12451 CAGCCTCACC TCTCTTCTGG ACAGGCCCTC CTTCTGACAA GGGCTTTGTT

FIG. 3-5

12501 CATTCTGCTC CCTCTGCCTA GAATGCCCC TTACTCTGTT CACTTAACCT
12551 CTGCTTATCG TTTAGATCTT TACCTGGATG GCTCAGAGAA ATATAGAAGT
12601 AATTCCTCAC CCTGAAAAAT AGGTTAGGTC CCTGTTTTAT GTTTTCATAG
12651 ACCTTTCCCTT TGAGGCTTTT TTTAAAAAAG TAGTTTTAAT CTCACATTTA
12701 TTCATGTGAT CATCTCCTTA ATGATATCTT AAGACCTCTA ATAGAACAAT
12751 TTGGTCATGG ACTGTGGGGT TTTTGCCCTT CATTGTGTCA GCACTGAGCA
12801 TATTGTTGGC ATAGGAGGGA TATTTGTTGA ATGAATTGCT AGAGGTGGCC
12851 AAGAGATATG ATGTAAGTCA GGCTTTTCCC TGCCCTTCCC CTTCCCTTC
12901 CCCACATCCT TCCTATAGCA GCCACCGTGG CTGCAGTTAC TGTAATGGC
12951 AAGACGGAAT CAGTTCGGA CATTGGGTG TTTTAGAAAA TTGCCTGCAA
13001 GTGTCAGGGT GATAAGTTAA AGCTTTGTCT TTTGCCCTCA GAGGAGCTAT
13051 CCCATAGTGA GTAGAAGCCA GAGAAGCTGA CCCCAGGAGT CTTCTTTCC
13101 AGCAGCAGGT CTTGAGCTGC ACTTCTCTGT AGCTACAATC CAGGCAGGAA
13151 CAAGCCCTAG GTACCTCCGG AGAGGAGGGC AAGAGAGGAA GAATGAGTTC
13201 AGCTACTCTA GCCACCAAAC TGATTATGAA TTGCCCTGAA ATCTGAAAAA
13251 TTTCAATTCC AATCGTAAGT TTGTTTTGTT TCATTTTGTT TTCTTAAATT
13301 GTATATTTGA AAGATGGCAT TAACTAAAGA TATATATTCA ATATAGAGTG
13351 GAAAAAATGG AATACTTGCA TAGTATCTTT TACTTATAGG TGATTTATGA
13401 TGGGGAGTGG GGTGGATAGG TTGGCAGTTC CCCCAGAAG TTGGAAATGA
13451 AGTTTGTCTT CTGTGAGTTG AACTAATTAG ATCCACAAGT AATGAAAGCA
13501 GTATTGTGTT GTAGTTAAGA GCACACTCTA GAACCAGATT GCTTAGTTTC
13551 AAATCCTGGT TCTGCCTTTT ATTATCTGTG TACTTTGGGC AAGTTACTTG
13601 CCCTTTGTGT GCTTCATTTT TCTCATCTAG AAAATGGAGA GGCCAGGCGT
13651 AGTGGCTCAT GCCTATAATC CCAGCACTTT GGGAGGCCGA GGCGGGCAGA
13701 TCACCTGAGG TGAGAAGTTC AAGACCAGCC TGGCCAACAT GGTGAAACCC
13751 TGTCTCTACA AAAATACAAA AATTAGCCAG GCATGATGGC GGGTGCCTGT
13801 AATCCCAGCT ACCCAGGAGC CTGAGGCGGG AGAAACACTT GAACCTGGAA
13851 GGCAGAGGTT GTAGTGAGCC AGGATTGCAC CACTGCACTC CAGCCTGGGT
13901 GACAAGAGCT AGACTCAGTC TAAAAAATAA AAAAAAATC AAACCTGGAGA
13951 TACAGGCTGG GTGCAGGGCT TACACTTATA ATATCAGCAC TTTGGGAGGC
14001 CTAGGCGGGA GGATTGCTTG AACTCAGGAG TTTCAAGATC AGTCTGGGTA
14051 ACAGAGCAAG ACCTCATCCC CACAAAAAAT CAAAAATTTA GCCAGGCATG
14101 GTGGCTCATG CCTGTGGTCC CAGCTACTCA GGAGGCTGAG GCGAGAGGAT
14151 TGCTTGAGCC CAGGAGGTTG AGGCTGCAGT GAACCATGAC TGCACCACTA
14201 CATGCCAGCC TGGATGACAG AGCAAGACCC TATCTCAAAA AAAAAAATAA
14251 AAAGAAACGA GCCAGGCGCG TTTGCTCACG CCAGTAATCC CAGCACTTTG
14301 GGAGGCCAAG GCAGGTGGAT CACTTGAGGT CAGGAGATCG AGACTAGCCT
14351 GGCCAACATG GTGAAACCCC ATCTCAACTG AAAATACAAA AATTAGCCAG
14401 GCATGGTGGC ATGCTCCTGT AGTCCCAGCT ACTCACTTGG AGGCTGAGGC
14451 ACGAGAATCG CTTGAACCCA GGAGGCGGAG GTTGCACTGG GCCAACATCA
14501 TGTCAGTGCA CTCCAGCCTG GGAGACAGAG CGAGACTCTG TCTCAATAAA
14551 TAAATAAACA TAAATAAAAA TAAATAAAAA TAAATAAAAA TAAAAAATAA
14601 TGGAGGCCAG CAGGCACGGT GGCTCACGCA TGTAATCCCA GCACTTTGGG
14651 AGGCCGAGGG GGGCGGATCA CAAGGTCAGG AGATCGAGAC CATCCTGGCT
14701 AACACAGTGA AACCCTGTCT CTACTAAAAA TACACAAAAT TAGCCAGGCA
14751 TGGTGGCAGG CACCTGTAGT CCCTGCTACT CAGGAGGCTG AGGCAGGAGA
14801 ATGGCGTGAA CCCGGGAGGC GGAGCTTGCA GTGAGCTGAG ATCGCGCCAC
14851 TGCAGTCCAG CCTGGGCGAC AGAGCAAGAC TCTGTCTCAA AAAAAAATAA
14901 AAAAATGGAG GTTGGGCGCG GTGGCTCGCG CCTGTAATCC CAGCACTTTG
14951 GGAGGTCGAG GCGGGCGGAT CACCTGAGGT CAGGAGTTCC AGACCAGCCT

FIG.3-6

15001 GGCCAACATG GTGAAACCTT GTCTCTACTA AAATTACAAA AATTAGCCAG
15051 GCACGATGGC AGGCACCTGT AATCCCAGCT ACTTAGGAGA CTAAGGCAGG
15101 AGAATAGCTT GAACCTGGGA GATGGAGGTT GCAGTGTGCT GAGATCGCGC
15151 CACTGCCCTC CAGTAGAGTG AGATTCCGTC TCAAAAAAAA AAAAAAAGAA
15201 GAAATGGAGA TACAAACTTA CTACCTACCT CCTTACAACC TACCCTCACA
15251 GTATTACTGT GAATAAAAGT GTGTGTAGCA CTGGGAACAC TATTACAGA
15301 GCACTCATGA ATGTTTGTTT TTTGTTATTA GTTACTAGAG AGGCAAATGT
15351 CTGCCAGGGC TGAATAATAT GTGTGAATTG GTGATTGTCG CACATATCTA
15401 AAGAAGTAGT TATTTTTTTC AATTAATACT TAGTTTAAAA ACCAATATAA
15451 GGCCGAGCGC AGTGGCTCAC ACCTGTAATC CCAGCACTTT GGGAGGCCGA
15501 GGTGGGCAGA TCATTTGAGG TCAGGAGTTC GAGACTAGCC TGGCCAACAT
15551 GGTGAAACCC TGTCTCTGCT AAAAAAAAAA AAAAAGTACA AAAATTAGCC
15601 AGGCATGATG GCAGGTCCCT GTAATCCCAG CTACTTGGGA GGCCGAGGCA
15651 GGAGAATTGC TTGAACCCAG GAGGTGGAGG TTGTAGTGAG CCGAGTTTGT
15701 GCCACTGCAC TTCAGCCTGG GTGACAGAGG GAGACACTGT CTCAAAAAAA
15751 AAAAAAAAAA ACCAAAACCA ATATAATAAA TAAGTGGCCA GCAATGAAAC
15801 AGAAAGTGAA AAGTTAGTGA AGCAAACTA GTACTGTATT CAGATAAAGA
15851 TGCTGAATCT AGATTTGGTC ACCAGAATAG GGTCTTTTGT GGCAACCTGG
15901 GCTAGTTTGG CTGACTCACC ACTGCCAGGA TGAATTTTCT TTCAGTGGCT
15951 ACTCATTTC CTTTATTTTA AGTCCATGCT CACAGAGCAA CCTTCTGATG
16001 CCTAATTCAG CTTCTGGGA TACTTAATAA CAGGAAGGGT CTGGAAGTAG
16051 TACCTGTATA GGGGATATGA GTGTTCTGAT TTTAATAGTC AATTCATAAG
16101 TGTACAGAGG GTTTGATAAA TGGTTAGGTC AGAACCATCA CAGAATGTCT
16151 ACACCTCTTT GGACATTAGG AAGGTCAAAA ACCTGAAAGG CCAAAGCTA
16201 GGCCTAGATT AGGGTCATTC ACCAAGAAAA CATCAGCCTT GAAGAGTTCT
16251 CTGGGTGGTC CACCAGTCAA CCTTCCTTTG ATCACACCTC CTTCTCGTT
16301 GCTTCTTTAA GCATTGACCT GTAATGGGTA TGAATTTTT TGCTCACCTA
16351 ACTCCTTCCT TTTACAGAGG AAGAAGTTGA AGCCCAGAGA GATTTAATGG
16401 CTTGCCTAAG ATCACACGCA GATTTTCTGT TAACCAGGGT GATTTTTCAG
16451 GTGTTCCCTG CCAGACGAGG GCTTTTTTCC TTGAATTGCC TAGAGATTTC
16501 TTGAGATATC CGAAGCATTT TTCCCAGTGC AGCCTGGAGA AGGTATGCCC
16551 TGCAACACA GCATTTGTTA CTCAATGTTA GACATTCAAT TTTCTAATTA
16601 GTATCATGGA GCAACAGTGG ATGATTATCT ATAAGGGGTT GCAATTCCAT
16651 GCTTATGTGC TTACAGCCCA TATAGACAAA TATCAGCTGT TAAAATGACA
16701 AGGCAGTAGA GATGTGGCCC CAGGACAAAG GCATACTCTG CTGTTAGTGA
16751 AACTAGTTG GCCAGCAAAT TTCACATGGG CATATACAGC GCGCACTGTA
16801 GACTTTAGGC ATTTATACCC ATTCAGAGAG CCAAAGTGGC AACTAAAGAT
16851 CAGCATTCTC TTTGGCATT TTTGGCATT CAGCTTTGCG TTCTGTAAA AATCACTGCT
16901 TGCTTAAATA CCTCTGATAG CTCTTCACTG CCTGTAGGCA ACTCTTAGC
16951 CTAGCAGACT TGGTCTTTAG TGCTCTGCCC CTACTCTCTT CCACCATTCT
17001 GGCCTCCTGT CTAATTGCTG CCCATATGTG CCATGCACTA GAGCTTACAG
17051 ACCTGCTCAG CGTTATATGA GCATACCATA CTCTTTATGC CTCAGTGCAT
17101 TTGCACATGT TGTTCCTTCA GGCCAGAATG CCTGTTACTG CCTGGCAATC
17151 AGCCTATTAG AGTCTGCCAA TACCATCCCA TCTTCTGTGG AGGAGCCCCC
17201 CGCCAAATCC ACCCATACCT CTCCCACCA ATCAGAGACT TCTTCTCTCT
17251 TTGTTATTCT CTTGTTATT CTCTTCATAC CTCAGTTATA TCCATTTCAG
17301 TATTTGTTTA CACATCTAGC ATCACTCTTA GAGTGTGAAA TTCTCCAAGT
17351 GTGGAGCCGT ATCTAGTTTG TCTTTGTATC CCAGAGCTTA GCAAAGTGCC
17401 TAGAATGTAG TGGGTGCTCA GAGTGTGTC TGGGTGAATG ATGTATTTGT
17451 TGAACGACTC TTTGGACACT TGAATAAAGT CCATCCAGTA TGCACCATTA

FIG.3-7

17501 CCATCTCTTC GCTCTACAAT ATTCTTTTAG GCAAGAGCTT ATCTTTTGAG
17551 GTGATAAGAT AAGCTCAAAC TTATGTAGAC TAAGACCTCA GTCTGTAAAT
17601 GTCATCCCTA AGTCTTAAAC CATCAAAACC AGGGCCTCAA GGAATGGCAT
17651 GCCTTCTGCA ACTGTAGCAA CCTGCTGTGC TTATTTTGCC GTGTTTTTCA
17701 TTTTCCCCC AAAAGCTAGA GTCCCTTCTC CCATGGGCAG TGCTGGAAGT
17751 GTGCTAACAA ATTCTTTCTC CATACTGCTT ACGATTACAA AAAAAACCT
17801 CAGCATCTCA TGCCAGACTT GAGTTAAGGT TGTTTTCTTT TGTGTGTGAG
17851 CTGTATTCTG GTCATGACTT CCTGATGATG CCCTATAGAG ATTTTGCTGA
17901 GATCAGAGGG TGCTCCACTG CCATCAGTAG CACTGACTCT TGCAGAAGCA
17951 CCGTTTCTGA AGTTGGCTAA TGTCATCCCT CACGTTTGTT TGTTTGAAAT
18001 TTGTTTTAGT TCCAGAGATA GCACTTTCAT GGAATGACGC TATCTTCTAG
18051 AATCACTTTT TTTTTTTTTT TGAGTTGGAG TCTCGCTGTG TCGCCAGGCT
18101 GGAGTGCAGT GGCACAATCT CAGCTCACTG CAATCTCCAC CTTCCGGGTT
18151 CAAGTGATTG CCCTGCCTCA GCCTCCCGAG GAGCTGTTAC TACAGGCGCA
18201 CACCCCACT CCTGGCTAAT TTTATGTGTT TTAGTAGAGA CGGGGTTTCA
18251 CCGTGTTGGC CAGGATGGTC TCGATCTCCT GACTTTGTGA TCTGCCTGCT
18301 TCAGCCTCCC AAAGTGCTGG GATTACAGGT GTGAGTCACC GCGCCTGGCC
18351 TAGAATCACC TTTTATACC ATAACGTGAG CACCACTGCC GCGTCACCAA
18401 GGAAAGAGAG AGGCAGCTAC TGTGGGGTTA CAAATGGGTA AGAGTGGCAC
18451 CAGGAAGGTG AAAGTCTCTA CTTAGCCAAG GCTTAACAAA ATGTCAATCA
18501 CCAAACATTT ATTTATTAAG CTACGTTTCA GATAAGAAGA TGAACAAGCT
18551 ATCTGTACAT TCATTTTCTC GTTTGTAACA AGGTAATGAT AGTGATCTAT
18601 CCTGCCTGCC TCTGAGGGTT ATTGTGAGAA TAAATGAAA TCAAGTGGAA
18651 AAGCACTTAG GAAAAAGAAA AGCATTGGTT TTCAATTGTT AGTGTGGATC
18701 AGAAACACTG GGGCTTGTTC AAAATGCAGA TTCTTAGCCC CAGTCTCAGC
18751 GATTCTGATT CTGTATATCT GAAGTGGGAC TCAGGAATCT TGATTTTCAA
18801 CAAGCTGACC AGAGGGTCCA ATGCTGCTAT TCCTTAGTTC ACACCTTCAG
18851 AAATATTACT GTAAATCAAA TGGCAAGAAT AAAATAGTTA TTTGAGGCAG
18901 TTTTAGTATG TTGGACCTGG AGTCCAAAGA CTTGGGTCAA ACTCCAGCTT
18951 TGTCAGTTCC TAGACCTGTG ACCTTAAACA GCAACCTTCT CTGTGAACCT
19001 TAGTTCCTC AGGAACGGCT CTGGTCACCT CCTGCTGTAC TCCATTGATG
19051 ACTCACCACA TAAGGCTCCC TGGGAGTCCC CCAAACCTTT GCTCTCTTAA
19101 CTCCTTTTAC AGCCTCCTAC ATCTCCTGCA GGTGCTGTCT TCTCCTCCTT
19151 TTTCCAGGCC CTGCTCTGAC ACAGCATTCA TTCTCCTCTG GGAAGGGTTC
19201 CTTCAATGTG TCTCCAAGCA CATCACACCC AGGAAGGACC CTGTGGCCAT
19251 ATCTGTCTAT CACCAGATCA AACTACGTGA AGGCAGGCAC TAGGTACTGT
19301 CAGTGCCAG CATAGGCCTG GCCCATACCA GGTGTCCACA GATGCCTAGT
19351 AAAGAAACCT ATGATTCAAG ACCCCCATGA TGAGCAACTA TAGCACTAGA
19401 ACAGTGATAA TAACTAATGT TTATAATGCA TCTTCAGTTT ACAGAGGGCT
19451 TTTGTACTCA TCATCTAGTT TAGTTCCTGC AACAACCTCT TGAGGAATAT
19501 AGCACAAGCA GGACAAGGGA AGCCCAGAGA TGTTAAATAA TTTATCCAAG
19551 TTTATGCTGC TGGGAAGGGC AGCACTGAAA TTAAGAGAAA AGTTTTCTGA
19601 GCTCAAATCC CATGCCCTTT CCTCAATGTG AGCTCTAGCA AGGTATTCAG
19651 GAATCCTGCC TCTACAGTTC AGAGCCTCAA ATTGCTGGGT ATGTTGAGTT
19701 CTTGTATCTG ATTTTCTAG ATTTCTGCC CACATTCTTA CTGTCTGGAT
19751 ATCAGGAAAG AGTTTATCAA ATGCCTGTGG AAATCCAAGA TAAGGTCTCA
19801 TGATGAGTAA CCCAGTGAAG ACATGAAGTC AAGTCTAACT AGTCACTACT
19851 ATTTCACTAC TGCTGACTCC TGATGATCAG CTCCTTTTCT AAGTGCTTAC
19901 TGTCCACTTA TTCCATCATC TGCCTAGAAT TTATGTGAAG GAATCAAAGC
19951 AAAAGGATCA TAAGGCTTCC TTTTCCAGT ATGTTTTTCC TCCTTTTGA

FIG. 3-8

20001 AAAC TGGGCC AGTTAGCTAT CTCCATTTTT ATTCATGAA TACATCCCCA
20051 GCGCCTGGTA TATAGTAGAT ATGGAACATT ACAC TTTGGA GATATTGCAC
20101 CCATTCTCCA GTTCTCCAA AGTTACTAAC AATGGTTCCA TCACTGTGCC
20151 AACATATTTT CTTTTTCAA TATATTGGGA AATAATTCTC CCAGTCTGAA
20201 AATCTGAACA CATTTCATGT GACTTGGTAT CCTCATATGT CTTGGGCTTC
20251 CAATTCTCCA TTCCTAGTTT CAAGTTCATG AACTGTAAAA CAAAGGATTA
20301 GACTAAATCT CTAAGTTCT ATCCAGATGC CAAATTCTTT TCTCTTTCCA
20351 TGATACCTAA GATAGATGCC AAATATTGTC TTTTACCTGG TGTTTGTGAA
20401 CATGACATCA CATTACAGGA GTAGCAGATA CTAACCTCTC ACTCTGTAAA
20451 ACAC TGA CTG AGTTCATGA GCCAGATACT GAAGTGAGCT TGTTACATA
20501 TGTTCTCATT TAATGCTCAT AACCCTGTGA AGCTGGGAAT TGCTGGGACA
20551 TTTTATTTAT TTATTTATTG AGACGGAGTC TGGCTCTGTC ACCTAGGCTG
20601 GTGTGCAATG GCATGATCTT GGCTCACC GC AACCTCCGCC TCCCGGGTTC
20651 AAGCGATTCT CTTGCCTCAG CCTCCGCAGT AGCTGGGATT ACGGGGCACA
20701 CACCACCACA TCCAGCTAAT TTTGTATTTT TAGCAGAGAT GGAGTTTCTC
20751 CATGTTGGCC AGGTTGGTCA CGAACACTTG ACCTCAAGTG ATCTGCCTGC
20801 CTCAGCCTCC CAAAGTGCTG GGATTACAGG CATGAGCCAC CATGCCTGCC
20851 CGGGACCCTT GTTTTAGAAG GATGACTGCT GCTATAATGT AGAAAGTGAT
20901 TTGGAAGAGG GGAGGAGTGG GGCACGAAAG ATGGTTAGTA GATGGGGGTG
20951 GTAATGCTTA CCTTTCAGTA TTTGGAGGCT TCGGAGTCCT CAAAAATTCT
21001 CTTCTTGAT TGGAGTCCTC CCAGCCAATA GAGGGCTTCA CACAAACAGT
21051 TTCTTGGGT TTGAATTGTT TGACCAGAGC TTTCTCCGA CAAAAGGTTG
21101 GGGTGATTCA TTCATTACC ACACCTTGCC TGAACATTCA CTTGGGGCTG
21151 CCGGTTATGA AGGCTATTGT TCTCCAGCCT GTCACAGACG CTTTGAAGAC
21201 CTGTGCCTCA GCTGGTTCTA AGGAGTCAGT TTGTTAGCT CCGTGCCAGG
21251 TTTCCAACCT ATGAAATGTG CTGGAGATTA ACACCTCTCC TGCCATTTTA
21301 TCCCTACTAT AATTGCCAGT CAAAGGATTC CTGCA GTTG C TCTGGCAGC
21351 CATAACTGAT GAATGTTCTG CCAGCTGCTC TGAGGACCTA GAAGAGCAGT
21401 TTTCTATCCA GGACCA GTT CCAAGGGTGG GAGGGTGAAA TATATCCTCC
21451 AGTGTGACAT TTCATCTCCC AGTGATGGGT GGCTTGGGCC CTTTGAAGTT
21501 GGCTCTGAGG AACCACACAC TTGGGTCTGA GCAGCCAGCA GCTTATCACA
21551 TCTGGTGATC AATCCTTCAA AGGTTCTCC TGAAGTCTGA ATTTTGGAG
21601 GTCAAATGGA TTCCACCTGG GAGGGGCTTC TGCTTCAACT CAGGACATGG
21651 GGAGAAGGCT GTTCTCTTC CAGGGGGAGG CAGTTTTCAT GGCATTGAGA
21701 TGTCCTCTCA CTTATTCCTC ACCCACCCAC CAAGTCCTTT GTAAGAGGAG
21751 TAGGGGGAGA GGAGAGCGCC TGCAGCCTCC TGCTACATT CCTAGACACC
21801 GACTCACTGA GCCCGTCGCC GCTGGAACAG CAGAGCTGTG TGAAATGTCA
21851 AGAGGAGTTA TGCTCATAGG CTCCTGGGCC TCAGTCTCTT TGTGGCTTGC
21901 ATATTCTTCC ATTAGTACTG TGTTATCAC ATGGAAATCA GAGGGTACAA
21951 TTAAGAGATA ATTTGCTAGT CCCAGACTTA ATTTGGGGCC CCCTTCTTGC
22001 CTGATTGAAT TACAGGGGAA CATAATAGAT TTTTGGTGAG AAATAGTTGT
22051 CTGTGTGGCT GGGAGAAAGA TTGCTCCAG CTCTCCAGCT GGGCAGCCCT
22101 TTCAGTATCC CGTATGTTAT TTCCCACTT CCAGCCACC TCACCTCTC
22151 TGTGGCCCTT GTGTGTCCC TCGGCTAGGA TCCTGACCTC CTGCTCAAGA
22201 GTTTAACTC AACTTGAGAC CCAAGGAAAA TAGAGAGCCC TCTGCAACCT
22251 CATAGGGGTG AAAAATGTTG ATGCTGGGAG CTATTTAGAG ACCTAACCA
22301 GGCCAGACA GAGAGAGTGA CTTGCTAAAG GCCACATAGC TAGCCACAG
22351 TAGTTGTAAC AATAGCTTA ATGATATTAA TGGCTAACAT TTATCAACCT
22401 TTAATGTGTC CCAGACTTTG TGCCAAGGGC TTACATGCAG TGCATTGTG
22451 CATTCAAACC CAGACAGTCT GGCTCTGGGC CCAGGCTGAG CTTTGGTATA

FIG.3-9

22501	GCATGGTAGA	ACGTTGTCTA	TAATGTCTAG	TCTGGGTTC	AATCCTGGCT
22551	TCATTCTCA	CATTTACAGC	TGAGTGACCT	CAGGCAAGTG	ATTTAACCTC
22601	CCTGTACCTC	AGTTGCTTTA	TCTGTAAAGA	GAAAAATCAC	AGCACTGTGG
22651	AATAGTGGGG	GTTAAAATTC	ATTCATACAA	GTAGTGCTGC	AAGCAATGTT
22701	TAATACAGGG	TGAGCACCTG	TTCAGTGCTT	CCTTCTTCTG	GCTGCCTCTG
22751	GGGCTAGAGT	GTGGTGTCTT	CGTGGTATAG	ATAGATAGAT	ATGGCTGAGC
22801	TCTGCACAAA	CACCAAGAGC	TGTTCTTCAC	TATTAGAGGT	AGTAAACAGA
22851	GTGGTTGAGC	TCTGTGGTTC	TAGAACAGAG	GCCGGCAAGC	TATGGCCCAT
22901	TGCCTATTTT	AATACGGCCT	GTGATTGATT	GATTTTTTTT	TTCTTTTTGA
22951	GACAGAGTTT	CACTCTTGTT	GCCCAGGCTG	GAATGCAATG	GCACGAACTC
23001	AGCTCACC GC	AACCTCTGCC	TCCTGGGTTT	AAGCGATTCT	CCTGTCTCAG
23051	CCTCTCGAGT	AGCTGGGATT	ACAGGCATGT	GCCACCACGC	CTGGCTAATT
23101	TTTGTATTTT	TAGTAGAGAC	AGGGTTTCTC	CATGTTGGTC	AGGCTAGTCT
23151	CGAACTTCCA	ACCTCAGGTG	ATCTGCCCGC	CTCAGCCTTC	CAAAGTGCTG
23201	GGATTACAGG	CGTGAGCCAC	CATGACTGGC	CTGATTGACT	GATTTTTTTA
23251	GTAGAGATAG	GGTCTTGTTT	TGTTACCCAG	GCTGGTCTCA	AACTTCTGGC
23301	TTCAAGCAGT	CCTCCCTCCT	TGGCCTCTCG	AATGCTGGGA	TTATAGGCAT
23351	GAGCCACTAT	GCCTGGCCTA	TATGACCTGT	GATTTTTAAT	GGTTAGGGGA
23401	AAAAAAGCAA	AAGAATGCTT	TGTGACATGT	GGAAATTACA	TGAAACTCAA
23451	ATATCAGTGT	CCCAGCCTGG	GCAACAAAGT	GAGACCCTGT	CTCTACAAAA
23501	AATAAAAAAA	AATAAGCCAG	GGCCGGGCGC	AGTGGCTCAC	ACCTATAATC
23551	TCAGCACTTT	GGGAGGCCGA	GGCAAGTGGA	TCACCTGAGG	TCAGGAGTTC
23601	AAGACCAGCC	TGACCAATAT	GGTGAAACCC	TGCTGTACT	AAAAACACAA
23651	AAATTAGCCG	AGCATGGTGG	CATGCGCTG	TAGTCCAGC	TACTTGGGAG
23701	GCTGAGACAA	GAGAATTGCT	TGAACCTGGG	AGGCGGAGGT	TGCAGTGAGC
23751	CAAGATCGCG	ACACTACACT	GCAGCCTGGG	CAACAGAGCG	AGACTCCGAC
23801	ACACGCACGC	ACGCACACAC	ACACACACAC	ACACACACAC	ACGCTGGGTA
23851	TGGTGGCCAG	CACGTGTGGT	CCCAGGATGC	ACTGGAGGCT	TAGGTAGGAG
23901	GATCACTTGA	GCTTAGGTGG	TTGAGACTAC	AATGAACCAT	GTTTATACCA
23951	CTGCACTTTA	GCCAGGGCAA	CAGTGTGAGA	CTGAATCTCA	AAAGAAAAAA
24001	AAAAAAAAGA	AAAAAATCTT	TCCATAAGTA	AATATCTGTT	GGAACATAGC
24051	CATGTCCCTT	AGTTTATGTT	TTATATATGG	CTGCTTTTGC	CCTATAATGA
24101	CACAATTGAG	TGGCCACGAC	AGTCTGTATG	GCCTGCAGAG	CCTAAGATAT
24151	TTGCTCTCTG	GCCCTTTACA	GAAAAAGTGC	CTTGACCTGT	GCTCTAGAGC
24201	CATATGTACC	AGGTTTGAAA	CTCAGCCTCA	CAGCTGGGTG	TGATGGCACG
24251	CATCTGTAGT	CCCAGCTACT	CTGGAGGCTG	AGGTGAGAGG	ATCACTTGAG
24301	TCCAGAAGGT	CGAGGTCAAG	ATTGTAGTGA	GCCATGATGG	CATCACC GCA
24351	CTCCAGCCTG	AGTGACAGAG	AGAGACCCTG	ACTCAAAAAA	AAAAAAACAA
24401	AAAAAAAATA	CACCCTCACC	ACTTATCAGC	TATTTGTCTT	GAGAATAGTG
24451	ACATAACCCC	TCAGAACCTA	TTTCCTAATC	TGTTAAATGA	GGCTGATGAC
24501	GTTTCCTCCT	TTTACTGGCA	ATTTAAACAT	GATGGATAAT	AAATGCTAAG
24551	CACTTAACAC	AGGGCCTAGA	AGATATTAAC	TGCTCAATAA	ATGGTAGCTT
24601	CTTAACAGTA	TTCAAACCCA	TGTGCTCTTA	TCACATGCAT	TGTTGTCCCT
24651	GTGTCCAGTT	GGTGGAATGG	GAAAAGGCTC	CCTTGTAACC	CCATCTACCA
24701	TCTTTATCAG	ACTTTCTGCT	CATGGTTCAC	AGTAAGAGAT	AGAAGCTGCA
24751	CGGTGACTTC	TGGCTCTTTA	CAATGGTGAG	CGGTGTGTGC	CTGGTAAGGG
24801	AGAGCTGATG	TCACTGCCCC	AAATCCAGTA	GTGAGATCTG	AGTGTTCCTG
24851	TTTCCTCCAG	CAGCCTTGCT	TTTTCTTTTA	CAATCCTGCA	GGCAGGGAGA
24901	CAAGGGCTTT	CTACATGGTA	GGCTCTGGTT	TGGTCATCGT	CACAACTGGG
24951	GGCTGTTCAG	GTGGGCTCCC	ATTCCAGATA	CCTAGGCTTA	TCAATCCCTT

FIG.3-10

25001 TTGGCACCCC AGGCCTTTTT CTCCTCATG CCCCATTTTT CAGTTTGAAA
25051 AGCATGGTTA TCACAGGACA AGTAGAAGAA GCTCCACTGT CCACTGAGGC
25101 CAATGGATGG TGTTCGTCAT GTGAACAETC AGTGAATAGT GAGTGAATGA
25151 GAGTAACCTG GGCTCCATCC TATTTGCAGA GAGCTTTGGA AAAGATTTTT
25201 CTCCTTAAAG AGCCAGAATG AAGCCTGGTA GTGGGAGAGC TCCAGCTCTA
25251 GAGTCACATG AGCCTACATT TAAATTCCAG CCCTGCCACT GACTCCCTTT
25301 TTGACCTTGA GTGAGTTACC TAATCTCTCT GTACCTCACT TTTCTTGTCT
25351 GTAGAGTGGG AATAATTCCT GTCTCAGAGA AATAAAAGAG TGCATATAGT
25401 GTTTGCCACA TGGAGACACA TCAGGTGTAG GTTAATACTC TGGCCCTTGT
25451 TTCCTTATTT GCAACACAGC CCTGCCCTGG AGTGGAAAGT GCACCTCCCA
25501 TTGGTCAGCT CTTGAGGCTG TCCCCAGGAC AGGCAGAGGG AGGGAATGAA
25551 TGGGAGCCCT AGTGCCAGGA CAGAACAGAT GGCAGCTCAG AGCTAGGATG
25601 GCTCTCTGGA CCTGTCTCTC CTACCAGAGG TCCCCCGTC TGGTGTGGCT
25651 CTTCTCTGGA CTGGCATCCT CTGCTTTTTT TTTTTTCCA CCTCCAAGCA
25701 GAATTACTGT CCTGTAGGCA GCTCCTCTGC TTGAGGACAT CTGGGGCCAG
25751 ATATGTTTAC ACTCTATCCT GCCTTGCCCT TCCCTGAGCT CAGGATGGAC
25801 GCTCAATTGG TCCCAGTTAT TGTCTGCAGC GCCTGCCTGC AGCCTCGATC
25851 CAGCCCAGCT CCACCCCTTG CCTGCAAGGT CTGTTTCTA ACAGCTGCTC
25901 CAACCACACA CCTCGGTTCT GCGGGAGCCC CTCCTCTTCC TCCCTCCCTC
25951 CCTCATTCAG GGGTGGGACT GAAGAAGAAG GCTAACTTGA CAGCAGCGCT
26001 TCTTCTTAG CTAGTCACCG GCCCTGCTC AAGAATGCCA GTGTGTGTGT
26051 AGCCTCCACA GAGAGGTCGT TTTCTCGGAG TCCAGAGGGG CCGCCTGAGC
26101 TTCTGAGAAC TAGGGAGGAG CCATCCCAGC CATGAGCCCC TGTGGGAATC
26151 TGCTGGGGGC CAAGTGGCCT GGAGTCCTCA GGCTCCGCA GCTGCTCCGG
26201 AGGGAGAGGT GAGCTCAGGG CAGCCTGCCT GCAGCCAGAG GTGCCGGGAG
26251 CCCCCGGCCT GTCATGGTGG CCATCTACAG CCGGCCTGAG GCAGTCACAG
26301 ACGGATTTGC AGCTGAGCCT GTCTATCTGG TGTGGGAAGA AGATGGGGAG
26351 TTACTTGTCA GTCCCGGCTT ACTTCACCTC CAGAGACCTG TTTCGGTGAG
26401 TTGGTCTCCG AGTTCCCTC TCCATCTCTC CTGGCCCTG GTCCTGAGAG
26451 GAGGGTGGTC TCCCTAAATC TCCTTCTCAC TTAGTCCTTT ACCATCGGTT
26501 CTGCCGGGCA GAAGCCAGCG GAGGTTATAC CCAAGGAGAA TCGGCCTTGT
26551 GAGGTACCCC CATTATGTCC TGGAAAGTGG GAGGGGAGGG ATATACCCAG
26601 AAGGAACTTC TTAGGGAGCT CCAGCTCCCC TTCTATCCCA GACAAACCTG
26651 AAGGAGCCTC CAAAAGATGC CACTGACCTG CCCATTGTAG ATGTTACTGC
26701 TTCCGGGGGG AATAGCCCAA ATAGAGTGCT GTTTCAGCT CTCACATGTC
26751 TTACCTGCGG GCCATGCTGC CTGCCAGGA ATTTGTCCCA ACAAGCAGGA
26801 TGGGCAGGTT TTGCCAACT GTGGAAGTGC GCAAGTCCTG GGTGTGGGTA
26851 GCCTGGTACA CAGTAGGCAC CTTATAAAGC TTTGTTCTCT TAATGGCAGG
26901 CACATTTGCC TCTGGCCTTG AAGGGCTTCT GAGCTCCCAG GTGAATGTAG
26951 TTGCTGGGGA AAGACCTGGG CGAGTGCTTC TAAGACTGGA GCAATGGGCT
27001 TTAGAGTGTT CCTGAGCTGC TGGGCCAGCC CCCACACCTC CTCAGTCCCT
27051 AGGCCTAAGT ACCTCCACGA GCCTCTCTCT GTGGGGCTTC TCAGAGGGAG
27101 ATGTGGAAAC TCTACCTCTA ACCTGGCTTT CTTTGCTCAT TGCCCCACTC
27151 CACCTCCCAT AGAACTCCC CAGGGGGTTT CTGGCCCTCT GGGTCCCTTC
27201 TGAATGGAGC CATTCCAGGC TAGGGTGGGG TTTGTTTTCA TTCTTTGGGA
27251 GCAGCCTGTT GTTCCAAAAA GGCTGCCTCC CCCTCACCAG TGGTCCTGGT
27301 CGACTTTTCC CTTCTGGCTT CTCTAAGCTA GGTCCAGTGC CCAGATCTTG
27351 CTGCCGGGAT ACTAGTCAGG TGGCCAGGCC CTGGGCAGAA AAGCAGTGTA
27401 CCATGTGGTT TTGTGGAATG ACCGGACCTT GGTAGATTGC TGGGAAGTGT
27451 CTGGACAGGG GGAAGGGGGA AGGGAAGTGG TCCTCAATGC TGACTCTACC

FIG.3-11

27501 AAGCGCCCTG CTAGACACTT TATCCTTTAA TCTCTCAACA GCCTAAAGAG
27551 ATTATATATC CCCATTTTAC AGATGAGGCA ACCAGTTTCA ACAGAGTTAA
27601 CATATGGAGC CTTACTGGGC AGCTTTTTCT GTCTTCCTGA CTTTCTCTCA
27651 TCCTTCAGGG GGCTGCAGGT TTGTTTTCTT CTCCTAGTGG AGAGGAAATT
27701 CTCAGGTTTG TTTTCCTCTC CTAGCAGAGA GTAAAAAAG GGATAGTTTG
27751 CCTGACTTGT TGAAGGTGTG GCTGAGATTG TTTTCTAAAG AGCCAATGGA
27801 AATTGATCTT GAGTTTAGGA GAAAGCTTTT ACATGTGGAA TTAAGATGCC
27851 AAGTGTTGAA GTAGCCACAT TTCAGGTCCT CATTAAATTC TCTTAATCCT
27901 GGGGAAGCAG CTTAGGAGAA GGGTTGTTCC TTTAGGAGCC AGGAACTATA
27951 CCCCTTTTAC CCTTGGAGAG GCAGGGAAGC CAGGGAGGAC ACAACTTCTC
28001 AGGAAGAGGA GAAGCTAGAG CAGATAGTGA ACTCTCAACC TGAACCTTTA
28051 AGGCCAGAG CACTAATGCC ACCCAAGTCC ACCTGCCGTT TGTCTGTTC
28101 TGTCACAGGC TTTCTGGAGA ACCTGATCTT CTTGCCCTA CCCCCAAGCT
28151 CCGTTTGCCC AGCTAGAGTC TGGGGGGTAC TGACTGACTT TCGTAGACAT
28201 TCTTCCCTTC CCCAAATAAG AGGCCACATT CCTGAAGTCA CTTCTGAAGA
28251 GATAGCTGCC ACACAGGGCT CTTTCCCCC AGGGAGGGAC CACCCAGACC
28301 CTCTGCTCTC CCAGGTATCC GTTACCACAT CACTACCTGG TCAGAAAGCT
28351 GTTCTGCCA TTAGCCCCCT CCTCTTTTAT TATAGGATAT CCTCAAGGGC
28401 TCCTCTTTGG GCCTCAGTTT CATCCTTGGC AGAAAGTAGA AGCTAGACTT
28451 CTTGGGCTCC TGAACAGGGT CTTGCTGGA TTCTGTGAAA CAAATTAAGT
28501 TCTTGACCCT AGGCCTCTGG GGGAGTACAA AGTCTATGGG AGTTCTGGGG
28551 CTGTGGTTGC AAGGAAAGTG ACGCAACCAG ATTCCATGGG GACATGATCA
28601 GCGGTGACAT GTGAGGGAGG AAGAGGGAGC AAGGGAATGA AGAATACAAC
28651 TTCTGTGTCC CATAACCCC TGCCTGACAG GCCATACATA CTCAGCAGAG
28701 AATGCACTGT CTTTCTTACC ACACTAGCGT GAGGAGTGAG CTGCAATTAC
28751 CACTGTGCTT CCAAGTAAGA AAATACCTCA AATTGGAATT TACAAAAGAG
28801 GTAAATTAGG GAGTGGCTTT TGTCGGACAT CTTTAAAGCA TTTTCTTTT
28851 TATAGAATTT CACTTAATGT CCAATACTGA TTTAATGAGC TTGGGTTTAC
28901 ACATTATCTC TTGAAGAAAA CAAATGAACC TTTGTGTTCC AAAGCAATCC
28951 ATGTTTAAAG GGAAAAAATT ATGCATAACT CTGCCAGCT TCACAGTAAC
29001 CTTTGGCAGG TGCCTTAGGT CCTCTGGGAC TCTTTTCTT ATCTGAAAAA
29051 TGAAGGACTT GGATCAGGTG AATGGTTCCC AGCTCTGCAA CTTATGTGGC
29101 TCCTCAGAGG CACACAAGCT CTTTTCATT ATTTGCCAAA TAATGGAGGC
29151 CCTGTCTTTA ACTGCAGTAC AACTACACAA AATACTTGAA ACTACAGTCT
29201 TCCTGGTTTT TGGTTGGAAC TGAATCAGTG CACTCTAGCA ACACTTATTT
29251 CTTGCTGTTT GTAGGCTTCA TTATGTGTTT GGTTAATTTT TTAACAAC
29301 AATAACATAT TCCATAATAA TTACAGCTTA ATTGGCAGAC TGTTCAGTC
29351 TATAGGATCT GCAGGAAGGA GGAGTAATAA AGGGATTTTT GACTGAGCTC
29401 TTATGGAACA GAGTCTCTCT AGGCCCTGT CATATCTGCC CTTCTGGGCC
29451 CTGGGGAAAA GTTGGCATCC CCAGTTGTGG TGCTCTCCAG GTGCCCTCAG
29501 GCTGTGGTGG AGGGAGCTTC CCATTCTCTC CTTAGCCCA CTCAATTCAG
29551 AGGCTAGGGG CTGAAAGAAG CTTCTCTACA ACTGGCTGTT CACTGGGAGG
29601 TTAAGGGATG ACCATCCAGC CAGGCCCTCC TCAGGACATG GGAGGGCTTA
29651 TGCTTTAACA TGTGTAAATC CACTGCAATA ATGACTGGTT CTTTTACCCC
29701 ATAAGGTTGA GAATTTACCT GTAAACATTT TTGTCTGAAG AATTTGGATG
29751 TAAGTGAGGG CTGGGCTCT ATCTTATCTC ACTTGGCTTC TCTCAGCACA
29801 GCACCTTGCC TGCTTGTCT TACACATCCT AGATGCACAG TAACTATTTT
29851 CTAATTATTA GAAATCTATT AGAATCAATT GATTCAGCT GGGCTTGGTG
29901 GCTCCTTCCT GTAATCCAG CACTTTGGGA GGCTAAGGCT GGAGGATCAC
29951 CTGAGTCCAG GAGTTTAAGA CCAGCCTGGG CAACATAGGG AGACCCTGTC

FIG.3-12

30001	TCTACAAAA	ATAAAAAATT	AGCCAGGCAT	GGTGGTGTGC	ACCTGTAGTC
30051	CCAGCTACTC	AGGAGGCTGA	GGCAGGAGGA	TCTCTTGAGC	CTGGGAGGTC
30101	AGACTACAGT	GAGCAATGAT	TGTGCCACTG	CACTCCAGCC	TGGGTGACAG
30151	AGTAAGACTC	TGTCTCTTAA	AAAAAAAAAA	AAAAAAGTTG	ATTTCTATTT
30201	GGATAGATAA	ATAATTCATT	TTAGGACCTT	TCTTTTTCAC	TTACAGAAAT
30251	CTGTTTCATT	CTGGGCTGAG	AAGCAGGTCC	ATATTGCTAG	GCATAGGAGA
30301	AAAAGGGGTC	TGTCTGCATT	TGCCCTTGGT	GGTCTCAAAT	TGGGGAGGGA
30351	AAGAAATGAA	CACTTACTGG	CTACCTTCTG	TGAGCCAGGC	ATCATGCAAG
30401	ACATCTGTAC	ATAATTTAAT	TCTCATAACC	CCATAAGATA	TTATTAGCAA
30451	TGTACAAGTG	AGGAAACTGA	GGCTCAGAGT	CATGAAGTAA	CTGGCCTTGG
30501	GTGACACAGA	TGGTAAATGG	CAGAGAAGGA	ATATGGATCC	AGGTCTTGAA
30551	AGAGAAAATC	TCAACTGATT	ATCTTTTTTA	AAAAACTCAT	ATGTTCTCTG
30601	CTGACTCAAA	AGGTCTCTGT	GTGGATCTGG	GTTGACCCAC	TGAACTGACC
30651	ATCAGGGTTC	CATGCACTTT	GTATCTGCCC	AAGCCCTCAG	AACCCCTCAG
30701	TAATGTTTTG	GAAGATGAGT	TTTGGAGGTT	GTCCTTAGGC	ATAGCCTCAG
30751	CGTATGTAGG	CCTCTAGGTG	ATCTCCCCTA	ACCTGAGGAT	TTCACTCAA
30801	TTCACTCTGG	CTCCTCAGGA	CAGTGGGATG	ACTGGTTCAG	ACCTCAGCTT
30851	TACCACCTCC	CAGCTGGGTA	CTCTTCTACC	TACAGCCAGG	GCAGATTTTG
30901	ACTTTCACTT	GAAACTTCCA	AAAATTGAAA	GGTAGAAAAA	CAGCCTTGGC
30951	TTTGGGAAGA	ACGTATGATG	TCCATGGCCT	CTAAGCATCT	GAGGTGGGAC
31001	ATGTTGAGT	AGCACCTTAC	AGTTCCAAAG	TGTGTTCTGG	GTTCTTTGTT
31051	TAAAAGAACA	GAGACTGCTG	GGGAATTGAA	CACTGTGAAG	TATATGAAGG
31101	AGGAGAATTG	TGCTATTTAA	CATTCACTAC	TTGGGCTAAA	GGAGAAGCAT
31151	CACGAAGTGT	TAACACTCAA	AGGGTCTTGA	GCTGTCAGGG	CTCCAGCTTC
31201	CTTATTTTCA	CAGGTGAGAA	TCCTGAGGCT	CAGCTGTTGA	GATGTGCTGT
31251	CTCACTCCGG	TGACATAGTA	CAGTGGATGT	GGCTTTGCAG	CCAAGCACAC
31301	ATAGCTTCAC	ATTCCAGCTC	CATCAATTAT	GTATTGGGCA	GCTTTGCAGA
31351	ATGATTTGAC	TTTAACTCTG	CTTTTCAGTC	TTCTGTAAAA	CAGGGATAAT
31401	CCTGCTACCG	TAGGGTTGTC	AGGATTAGAG	ATAATATAAA	TAAGGTACCT
31451	CATATAGGAC	CTGGATTATG	GCTGGCATTG	AATAAATAGT	AGCTGTTAAT
31501	TGATAGCTAA	GCTAGAACTC	TGAAGTCTAC	CATGGCAACT	TCTTAAGTGG
31551	TCTGAGAACC	CAGTTGTGTT	CTGTGGCAAA	ACACAGCTTA	GGGATCCATA
31601	CCCAGCCCTC	CTGTCAGCTG	TTACCTTCC	AGTTCTTCAG	AGACATGTGT
31651	GGCAGTGACT	TTGGCCACAT	AGCTGGCTGT	GCCCTTTAAA	GGCATTCCCT
31701	GACACAGATA	TGTGGACTGG	TGACGTTGCT	CTCCAGCCAG	GTGTTCTTCC
31751	CAGCAGGCTG	GCCTGGCTGT	CTCCTGCATG	CCTGTACTTG	TTTGTCTCCC
31801	TGCTCCCTCT	CCTGGGCCTG	GCCAGAGCTA	CTTGCAGCAA	ACAAAAGCAG
31851	GATATTGGCA	ATGGAAAGGA	GGGTGTGTTT	TGGTGCTCCC	ATGCCCTGCG
31901	GCGCACATAC	CATTGCAAGG	GCGTAACAGA	GCCCAGGCCT	GCAATTTGGGT
31951	GCAAATAAGT	CTGCACACAG	AAGAAAAGAA	GGACCTGGTG	ACCAGGAGCC
32001	ATGGAACCTT	TGTGCTCCCC	TACCTGGGCT	ACTGGTCTCT	GCCACTCCTA
32051	CCATTTTCAG	TTTGGAAATA	TTTGTTAAGG	CTTTGCTCTT	CCAGGTCTCT
32101	TGCTTGGTGC	TGAGTCTACC	AAGAGTAAGT	GGGATGCTGT	TTTTGTCTCT
32151	AGGGAGCTAA	CAGTCTAGTG	AAGAAGAAAG	ATGGTTGCCC	AGGAACCTCT
32201	AAGTCAGAAG	GCAGGAGGCA	AGAAGGAAGC	CCCTGCTCCT	ACTGCCAGCC
32251	CTCTGTTGGG	CACCCCATAG	TTCTTCAGAA	CCACATTTAA	TCCTCACTGC
32301	AGGCCAGGCA	TAGTGGCTCA	CACCTGTAAT	CGCAGCACTT	CGGGAGGCCA
32351	AGGCGGGCAG	ATCACTTGAG	GTCGGGAGTT	CGAGACCAGC	CTACCAACA
32401	TGGGGAACC	CCGTCTCTAC	TAAAAATAGA	AAAATTAGCC	GGGTGTGGTG
32451	GCATGCGCCA	GTAATCCAG	CTACTCAGGA	GGCTGAGGTG	GGAAAATCAC

FIG.3-13

32501 TTGAACTCGG GAAGCAGAGG TTGCAGTGAG CCGAGATTGT GCCACTGCAC
32551 TCCAGCCTGG GCGATAAGAG CAAAATTCCA TCTCAAAAAA AAAAAGAAAA
32601 AAGAAAAAAT CCTCACTGCT ACCTTGAAAG TAGGTGATGA CATTGCCATT
32651 TCACAAATGA GAAGTGAAGG GGCTAGCCCA AGATCACTTA GGTGGTAAAT
32701 GGTGGTGCTA AGATTAGAAC CTCAGATCAT CTAGGGAAAA ACACAGATAT
32751 GCACAGAGTT AAGGGGACCC AGGGTATTGT TTGTCCTCTT GTTTCACAGG
32801 TGGGGAAACA ACCCAGAGAG GGAAAGGGGC TTGTCCAAGG CAATTTAGCA
32851 CCCAAGAACT TGAACCCATA TCTCTCTCCT CCTCATTTAG AGTCATCCC
32901 ACATGTATCT TATATTGAGA GGAGTGTGAG CCACATACCA AGAACAGTCT
32951 TCCCCTCTGC CTCCAACCTC ACTGTGCAGT TTTGAGACAC TTCACAGCCA
33001 TACTCTTCAT GCCATACCCA GCCCTTAAGA CCCTGAAGTT CCCCTTCCAT
33051 AAGACAAGTA GGAAAAGCTA TAGGGTAAAA ATAGCCATCA GTGTTTGTTG
33101 AGCACCAGG AGGAATTGGG CACTCCAGAA AGATAAAGGG ATTCTCAGGG
33151 ACTTGCTTCT CTAGACTTCC CTAGCTCAGC TGCTTCAACT CATTCTGCC
33201 CCTCTTCTCT ACCTCCCGCA GTGCTCAGAA GTAGTAGAAC TCACTGTGGC
33251 CTCTCACCTT GCATTGTTGA GTTTTATTTA GACTTTCTCT TCCTCAACTC
33301 TTCATAAGCT CATGAAAGGT GAAGTAGGGT GCCCTGTGTA TTTATCTTTT
33351 ATATCTGCAG TGCTTAGCAA GTTATAATAA TGCACTTGCC TGGCAAAAGG
33401 CTTTCTCTCA TACATTAGCT TATTTCTCTT TCACATTGGC TCTTTGTAGT
33451 AATAGGATGC TATTAGTTAT TTTCAATGAG AGAAAGCTAC TAAGAGAAGT
33501 TGTCCAGCTA GTGACAGTAA GTGGCTGATA AAGTGAGCTG CCATTACATT
33551 GTCATCATCT TTAATAGAAG TTAACACATA CTGAGTTTCT ACTATATTGG
33601 GTCTTTTTTT TTTTTTTTTT TTTTTTTTTA GAGACGGAAT CTTGCTCTGT
33651 TGTCCAGGCT GGAACGCAGT GGTGCAATTT TGGGTCACCA CAACCTCCGC
33701 TTCCCAGGTT CAAGCGATTG TCCTGCCTCA GCCTCTGAG TAGCTGGGAC
33751 TACCAGTGCA CGCCACCAGC CCCGGCTAAT TTTTGTATTT TTAGTAGAGA
33801 CAGGGTTTCA CCATGTTGGC CAGGCTGGTC TTGAACTCCT GACCTTGTGA
33851 TCTGCCCGCC TCAGCCTCCC AAAGTGCTGG GATTACAGGT GTGAGCCACC
33901 GCGCCCTGCC TATATTAGGA CTTTTATATA AGCTATCTCT AGCTAGCTAG
33951 CTAGCTAGCT ATAATGTTTT TTGAGACAGA GTCTGACTCT GTCACCCAGG
34001 CTGGAGTGCA GTGGCGTGAT CTCGACTCAC TGCAACCTCC ACCTCTGGG
34051 TTCCAGTGAT TCTCCTGCCT CAGCCTCCCG AGTAGCTGGG ATTATAGGTG
34101 CATGCCACCA CGCCCAGCTA ATTTTTTGTA TTTTGTAGTAG ACCAGTTTC
34151 ACCATGTTGG CCAGGCTGGT CTCGAACTCC TGACTTCAAG TGATCCACCC
34201 GCCTCGGCCT CCCAAAGTGC TGGGATTATA AGCATAAGCC ACTGTGCCCA
34251 GCTGCTCTCT ATATTTTTAA TACATATTAT TTCCATTAAT TTTACAGCA
34301 GTTCATTTTA TAGATGAGGA AACTAGGCCA GAGAAGTAAA ATATCTTGCC
34351 CAAGATGATG TAACTAGTAA GTGGCAGGAT CAAGATTCAA ACCAAGCAAT
34401 GTTCAAACCT CTTGGAAGCA AGAATGTGGC CACTGTGGAA GGTGCAAGGC
34451 CTTGACAACA AGAATAGGGA AAAGAAGGAA CTAGAAGGAA AGAGATGGCA
34501 TGGGCTCAGC AGGCCAGGGA GCTCTTAGCT GTGTGTGTTG GGAAGCTCAG
34551 AAGGGAGGAA GAGGTTGTCT GTGCAGGTAA GTCCTGAGAA CACACCAGAC
34601 TTTTGAGAGG TGGAGCTTCA TAGCCAGGTC ATTAGGGGAG AAGGGAGCTA
34651 TAGATTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTAG AGACGGGGTC
34701 TTAATATGTT GCCCAGGCTG GTCTTGAAGT CCTGGGCTCA AGTGATCCTC
34751 CCACCTCAGC CTCCCAAAGT GCTGGGATTA GAGGCATCAG CCACCCCGCC
34801 CAGCGAGCTA TGGATCTAAC ATGTACATCT TACACAGTGC TAATAGAATG
34851 TTGGGTTTCT TCCCAATAT TTTATTTTGA AAAAAAATTC AAATATATAG
34901 AAAAGTTGAA AAATGTAGTT CAAAGAACAC CTACATACCT TTCACATAGA
34951 TTCATGATTT GTTAATGTTA TGCCACTTGT TATATATCTC TCTCCCTCTC

FIG.3-14

35001 ATCTGTATAC TTTTATTTAT TTATTTTTCG TGAACATTTT CAGAGTAACT
35051 TAAAGGCATC TTGATTTTAC CCTTGAACAG TTCAATATGT TTCTGCTAAG
35101 AATTCTCCTA TATAAGTCAG ATATCATTAC ATCTAAGAAA ATTCACGGCA
35151 ATTTTACAAT ATAATATTAT AGTCCAAATC CATATTTTCT CAGTTGTTCC
35201 AAAAAATGTT CATGGCTGTT TCCTTTTTTA ATCTAAATTT GAATCCAAGT
35251 TTGAGGCATT GTATTTGGTT GCTGTGTCTC TAGGGTTTTT AAAATCTGTG
35301 CCTTTTCTTC TCCCATGAC TTTTGAAG AGTCAAGACC GGTTATTCTT
35351 ATAGAATAAC CCACATTCTA GATTTGCCTG ATTAGTTTTT TTATACTTAA
35401 CGTATTTTTG GCAAGAACAT TACATTGGTA ACGCTGTTGG TGATGGGTCA
35451 GTTTTGAAGA GTGGAGATGA TTAACTGCT TTTGTTTATT GAAGTATCTG
35501 TCAAGACCAG AGATCCTTAA CTGGTGCCAT AAATAGGTTT CAGAGAATCC
35551 TTTATATATA CACCCTGTCC CCCACCTAAA TTATATACAC ATCTTCTTTA
35601 TATATTCATT TTTCTAGGGG AGGCTTCTTG GCTTTTATCA AATTCTCAGA
35651 GGGCCCCAAG ACCCAAAGAG GTTATGAAAC ACTAGTCTGT CCACTGAGGC
35701 AGGCAACACA GAGCTGGTTT CTGGGGCCTT GTTCAGTCTG AACCAGCTTC
35751 CCTTGGGGAG ATAGCACAAG GCTGTAACCT TGCCCCATCT TGGCTTTGGA
35801 TCAAAGAGGA CTGTCCATTT TGTGTGCTA CCTAGGAACC AGGGACAGCT
35851 TATGTGGCCT GGTTCAGGG ATCCAGGAGA ATTTCACTTC TTGTCTTGCC
35901 TTTCAAGGTG TCAGATGCC AGGATTCCTT CACCAACTGG TACTATGAGA
35951 AGGATGGGAA GCTCTACTGC CCCAAGGACT ACTGGGGGAA GTTTGGGGAG
36001 TTCTGTCATG GGTGCTCCCT GCTGATGACA GGGCCTTTTA TGGTGAGTGA
36051 ATCCCTTCAT ATCTGCCCCT CTTGGTCTTC AGAGTCCATT GACAGTGCCT
36101 CCAGTTCCTT GTGGCCTGTT AATCTTTTAG TCTTTCCATC AGCCAGGGCA
36151 TCTCCCTTTA TTTATTCATT CATTCAACTA GCAGGTATCA ATTGAGCACC
36201 TACTAAGTGA AAGGTAAAGT CCTTCCCTCA AAGACTTAAT AGTTGAACGT
36251 TGGGAGTGGG AGGAGAGGCA GGCAGAGAGG AGACACAATA TAGTTGGATA
36301 AGGACCTCCA AGGAGAGTGT TACAGGCTGA GAGGAGGATA TACTTAGGTT
36351 GTCTTTAGGG AATCAGAAAA GGAGACTCTG GAATAGGCTG GCAGAGAGAG
36401 GGGCTACCTC CTATACCTGC TCTGGACAAA CGACTTTAAG CATAGTGACA
36451 GATTTGCCAA CCCTGTATTG GAAGAACTGA TCTTTTTTAG TGGGGATGAT
36501 TACTTCTGGG GATTTCTTCT CATAACTGAG ACCAAAACAG TTTTGTGCAG
36551 TCTCAGAAAT GACAGGAGGT ACCAATCTGA CACTTCCTTT GGAAGCTCTA
36601 GGGCAGAGAG TGAAAGAGTG GATTTTGACG GGGGCCTTGC TTGGAGGTCA
36651 TTCACCCACC CCTGTCCTCA CTCCAGCAAC AGTGATAACT CACTTCCTTC
36701 CTCCCTTTGT ACACCTTCT CCCACCTGC TCACAGGTGG CTGGGGAGTT
36751 CAAGTACCAC CCAGAGTGCT TTGCCTGTAT GAGCTGCAAG GTGATCATTG
36801 AGGATGGGGA TGCATATGCA CTGGTGCAGC ATGCCACCCT CTAAGGTAA
36851 GATAGTGGTC CTTTGTCTAT CCTCTCCAT ATAAGAGTGG CTGGCGGGGA
36901 GGGACAGTGG CAGGGTGAGT TGGGCAGAAG GAGTGTAGG GTAGTCAGAG
36951 CATTGGATTG TTACCACAGC AGTGCTCTTA ACCAGCTCTT TAAGTTGTA
37001 GCAGAATGAT TTACACATGT CTCTACCCTT TTTCTTACC AACCTTGAA
37051 ATGTCTTCAC TCTGCCCTGC AATCCTCCCA GTGGGAGGCA CTCTTCAAGG
37101 ACGATCCCAG AACATTAAAG TCAAAGACCC CTTAGAGCTC ACCCTGTCCA
37151 ACCACCTTGG TTGATAAAAG AAGTCAGCCT GGGGCCCATG GAATAGAATA
37201 GTACAAGGGC AAGGTTCTCA TTGTGAGTCA AAGGTAGAGT GAAGAGAACC
37251 CAGACCATCT CACCCCAACC CAGGCCAGTG TTTTCCAAA TATACCACTT
37301 GCTGCAGATC TAGCTCAGCA CCCCAGTCC CAGCCCACCC TGAGAACCCA
37351 GGCTCCTCAT TCTGAGCAGC CAGCTAGAAT CATGACAAAG AGGGTGGTAG
37401 TGAGACTATG GGTACTGTTG CTTAAAGCCA CATGGTGCAG TGGTTGCTGG
37451 GGGGCTTCTG TGTGGGACTC TAGCATCTTA TTCCCCCTG TGCCCTCTCC

FIG.3-15

37501 CCAGTGGGAA GTGCCACAAT GAGGTGGTGC TGGCACCCAT GTTTGAGAGA
37551 CTCTCCACAG AGTCTGTTCA GGAGCAGCTG CCCTACTCTG TCACGCTCAT
37601 CTCCATGCCG GCCACCACTG AAGGCAGGCG GGGCTTCTCC GTGTCCGTGG
37651 AGAGTGCCCTG CTCCAACCTAC GCCACCACTG TGCAAGTGAA AGAGTAAGTA
37701 TTTTGAGAAC CCTTCAGCAG GGGTTCTTGA GCAGAGTCTG TAAATGGGCC
37751 TCAGAGGGCT TAGACCTCCA AAGTCTCATG CAGAACTCCC TTTATTCTCA
37801 TCTCATATCT TTCTCCTGGA CCCCCTATG CTGTAACCGT ACCTGGGCCT
37851 TGGCACTTAC TGTTCTCTCT GCCCAGGCTA CTTCTTACCC GATACTTAAG
37901 GCAAGAATCA CTCACCTTTC AGGTGTCAGG TTTCAGGTCA TGTTTGCTCT
37951 TTGAAATCAT CTGGCTTGAT TATGTGTATT AGTTGTTTAT CTTCTATCCC
38001 CTCCACTAGA ATGTAAATTC CAGAAGAAAC TTGCTGTCTT ATTCACTGCT
38051 GCATGCCCAG GGCTTGGAAG AGTACCTGGC ATATAGTAGG AGTTGATTGA
38101 TTATTATTTT GTCAGTCGAG AGAATGAATG GAGAAAATGT GGTCCATGGC
38151 CAAAAGAAG TTAAGACCCT ATCCTAGATT CAGGCCAGAG ACCAGATGGA
38201 GAAAGAGTCT GTGTCTATCT AATACCAGTA ATGTCGTACC TCTGGCCGCT
38251 TACCATGTAA ATATTGATTG TGTATCTACC ATGTGTTGGA CACTAGGCTA
38301 GTGCTTGAC AGCAGGTGAA AGATACTAGA GTTTGGGAAG TCAGGAGGAG
38351 CTAAGGTCTG TTCTACAACC TTATTAGATG AAGAGGAGAG GGAATTGTGT
38401 TCAGGGCAGA GGGAGAAGCA TTTCTCCAAA AGTAGGAGTC TTAATCATGT
38451 CTGATGTAGG TTGAGTGTGG CCAGAAAAGG GGCTGTTAAG TATAGAGGGC
38501 CTGGATTATG AAAATCCAGC AGATCCATTG AGAGTTTAAG CAGCAAGGTG
38551 TTGTGACCAA GTTAACATTT TAGAAGGATC ACTGGTATGG AGGTTGGATT
38601 GGAGAGGGGA AAGCCTAAAG GTATAGAGAC TAGTTAGGAA GCTATTGTAG
38651 GCTGGGCATG GTGGTTCATG CCTGTAATCT CAGCACTTTG GGAGGCTGAG
38701 GTGGGAGGAT TGCTTGAGGC CAGGAGTTGA AGACCAACCT GGCCAACATA
38751 GCAAGACCCC GTCTCTGTTT TTCTTAATTA AAAGAAAAGT CCAGACGTAG
38801 ACATAGTGGC TCACGCCTGT AATGCCAGCA CTTTGGGAGG CCAAGGTGGG
38851 CAGATTGCTT GAGGTCAAGA GTTTGGGATT AGGCCAGGCG CAGTGGCTCA
38901 CGCCTGTAAT CCCAGCACTT TGGGAGGCCG AGGTGGGCGG ATCACAAGGT
38951 CAGGAGATCA AGACCATCCT GGCTAACACA ATGAAACCCC GTCTCTACTA
39001 AAAGTACAAA AATTAGCCGG GCATGGTGGC GGACGCCTGT AGTCCCAGCT
39051 ACTCGGAGG CTGAGGCAGG AGAATGGCGT GAACCTAGGA GCGCGAGCTT
39101 GCTGTGAGCA GAGATCACGC CACTGCACTC CAGCCTGAGC GACAGAGCGA
39151 GACTCCATCT CAAAAAAAAA AAAGAGTTTG GGATTAGCCT GGCCAACATG
39201 GCAAAACCCC ATCTCTACAA AAAGTACAAA AAAATTAGCT GGGTATGGTG
39251 GTGCGCGCCT GTAATCCCAG TTAATCAGGA GGCTGAGGCA TGAGAATTGC
39301 TTGAGCCTGG GAGGTGGAGG TTGCAGTGAG CCCAGATCAT GCCACTGCAC
39351 TCCAGCCTGG ATGACAGAGT AAGATGCCAT CTCAAATAAA AATTAATAAC
39401 AAAGTTTAAA AAAAAAATAG AAGCTATTAC CGTGATCCAG GTAAGAGATG
39451 TGAATAACTA CAATGATGGA AAGAAGGCAG AGTTCTTAGA GATGGGAGTA
39501 GGAGAGATGA GGGAACCTCA GATTGGGAAG ATGATGTTCA AGTTTCTGGC
39551 TTAGGCCACA GGGTGAGTGG CAATTCCCTT CACTGAGATG GGGCATCCTG
39601 GAAAAGGTGT TGCCTTTCTG TGTGGGTATC CTGGGCCCTT TAGGGGCCAC
39651 TGGTGGCCTG GGACCTGGTA AACCTTCCCT GCACAAGCAG AATTGGTCAA
39701 GCAGGTTTTT AGGACATCTT TACCCTGCCT CAACTCTTGT CTGGCCAGG
39751 GTCAACCGGA TGCACATCAG TCCCAACAAT CGAAACGCCA TCCACCCTGG
39801 GGACCGCATC CTGGAGATCA ATGGGACCCC CGTCCGCACA CTTGAGTGG
39851 AGGAGGTAGA GTGTGTGTCT AATCTGTCTT GTGAGGGTGG GACATGGAAC
39901 AGATCCTCTG GGAAATCAGG CTGTAGCCTT TACCTTTTCC TACCCCCAGC
39951 CCATCTCTTT GTCTTAGCAT TGAGCCTGTG ACCACTGGTG ACCTATTCA

FIG.3-16

40001 GCGTAACAGG TTCCACAGGT AGCAGGGATG GTTGATGGAC GGGAGAGCTG
40051 ACAGGATGCC AGGCAGAGGG CACTGTGAGG CCACTGGCAG CTAAAGGCCA
40101 CCATTAGACA AGTTGAGCAC TGGCCACACT GTGCCTGAGT CATCTGGGTT
40151 GGCCATGGGT GGCCTGGGAT GGGGCAGCCT GTGGGAGCTT TATACTGCTC
40201 TTGGCCACAG GTGGAGGATG CAATTAGCCA GACGAGCCAG ACACCTCAGC
40251 TGTTGATTGA ACATGACCCC GTCTCCCAAC GCCTGGACCA GCTGCGGCTG
40301 GAGGCCCGGC TCGCTCCTCA CATGCAGAAT GCCGGACACC CCCACGCCCT
40351 CAGCACCTG GACACCAAGG AGAATCTGGA GGGGACACTG AGGAGACGTT
40401 CCCTAAGGTG CCACCTCCCA CCCTGGCTCT GTTCTGTCCT ATGTCTGTCT
40451 CTCGGATGAA GCTGAGCTGG CTTTCAGAAG CCTGCAGAGT TAGGAAAGGA
40501 ACCAGCTGGC CAGGGACAGA CTATGAGGAT TGTGCTGACC CAGCTGCCCC
40551 TGTGGGGATC ACAGTTTACA GCCAGAGCCT GTGCGGACCC AGCTGTCTGC
40601 CAGGTTTCCT TAGAAACCTG AGAGTCAGTC TCTGTCCACT GAACTCCTAA
40651 GCTGGACAGG AGGCAGTGAT GCTAAACCCT GAAGGGCAAC ATGGCCTATG
40701 GAGAAAGCAT GGAGCTCAGA GCCTGGAGTA CGGGCACAGA TAGGATTGAA
40751 TAAATTGTGT AGAAAGACTT TGAAAACAAT AAAGCAAAAG ATGAATGAAC
40801 GTTTTTTTTA GACTTGAGGG ACCAACAACC CCCAAACCCC AGATTCTGCC
40851 AGGTCCATGG GGAAGGAGAA GTTGCCTTGA GTGGAAGCCC CAAGTAGGGA
40901 GACTTACAGA AAAGAAGTCA AGAGCACTGG CTCCCAGGCA GAAATACTGA
40951 TACCCTACTG GGGCTTCAGG CTGAGCTCCT CCCTTCACAA ATCACTTCAT
41001 CTCTCTGAGC CTGTTTCTGC ATCTGTGACA TAAGATGGTA AGATAAAGGT
41051 GGCTGTCTCA CCAATTATGT AAGGATTAAA TGTGAAAAG GACATAAAGT
41101 TGTATAGTGC TGCCATAGGG ACAGTGTTCA GTAAACGTGA CACATTCTTA
41151 GTATCACTAA GAATCAGGTT CTTGGCCAGG CACCGTGGCT CATGCCTGTA
41201 ATCCCAACAC TCTGGGAGGC CTAGGTCGGA GGATGGCTTG AACACAGGAG
41251 TTTGAGACCA GCCTGAGCAA CATAGTGAGA CACTGTCTCT ACAAAAAAAA
41301 AATAATAATA ATAATTGTTT TTAATTAGAT GGGCAGGGCA CTGTGGCTCA
41351 CACCTGTAAT CCCAGCACTT TGGGAGGCCA AGGCCGGAGG ATTGCTTGAG
41401 GCCAGGAGTT CAGGAGCAGC CTGGGCCACA TTCCTGTCTC TACAAAGAAT
41451 AAAAAAGTTA ACTGGGCATG GTGGCACATG CCTGTAATCC CAGCTACTCA
41501 AGAGGCTGAG GAGGAGGATT GCCTGAGCCC AGGAGTTCAA GACTGCAGTG
41551 AGCCTTGATC ACACCACTGT ACTACAGCTT GGGCAACAGA GTGAGACCTT
41601 GTCTCCAAAA AAAAAAGTTT GTTTTTTTTT ATCCACTCTC CTCACCAAAC
41651 AACTGAGTA AGTTAGAGCC CTCTCAGCTG GCATGTGTTG GAAACAGTGC
41701 CCTCTCATTA AAGTGCTGCC CTCCTCCCA TTGCCTCTTG GCCTTGGTCA
41751 GTATGATGAA ATTAGTGGGA GGCAGGGCAA CAGAGGGCAG GGAAGAGCTA
41801 GAAATCCATG GCCTGGAAAA GGAAGATTG GGGAGTGGCC AGGTATCTGT
41851 AGAGCCACCA TGCAGAGGAG GGGGGCAGCT AGCCTTGTGT GCTCTGGTGG
41901 GCATGGTCAG CAGGAGGCAG AGCAAAAGGA CAAGGGTAAG TAAACCTGTA
41951 GGTGGGACA AGCCAAGAGC CATCCAGCGT CAGTCCTCTC TGGGTAGCCC
42001 AAGTAAAGCA GGAGCATACC CCAGAGAGAA AGTTCGCAGG GCTGTTCAAC
42051 TGCAGTGCTG TGGACTTCAA CCTTCTTGT CCTTCTTCAG TAAGTAAAAA
42101 TAACAGTCAT TGACCATGAC TATTATCGAC CGCTTTTGAA AATGTAAACA
42151 TAGTGACTTT ATTGCTGTAA AAATCATACG TGTTTATCAT CTTAAATTC
42201 AGGAAACATG GACAGGTACA AAGATGTGCA AAATATCATC CAAATCCCA
42251 TTTGCTGGCC AGGCACGGTG GCTCACGCT GTAATCCAG CACATTGGGA
42301 GGCCGAGGCG GGCAATCAC TTGAGGTCAG GAGTTTGAGA CCAGCCTGGC
42351 CAACATGGTG AAACCCTATC TCTACTAAAA ATACAATAAT TAGGCTGGGC
42401 GCAGTGGCTC ACGCCTATAA TCCCAGCACT TTGGGAGGCC GAGGTGGGCG
42451 AATCACAAGG TCAGGAGTTT GAGACTAGCC TGGCCAATAT GGTGAAACCC

FIG.3-17

42501 CATCTCTACT AAAAATACAA AAATTAGGGC CGGGTGTGGT GGCTCACGCC
42551 TGTAATCCCA GCACTTAGGG AGGCCGAGAC AGATGGATCG CGAGATCAGG
42601 AGTTCGAGAC CAACCTAGCC AACATGGTGA AACCCTATCT CTAATAAAAA
42651 AATACAAAAA TTATTCGGTT GTGGTGGCAC ACGCCTGTAA TCCCAGCTAC
42701 TTGGGAGGCT GAGGCAGGAG AATCTCTTGA ACCTGGGAGG CAGAGGTTGC
42751 AGTGAGTGGA GATCCCGCCG TTGCACTCCA GCCTGGGCGA CAGAGTGAGA
42801 CTCCATCAAA AAAAAAAAAA AAAAAAAAAA AAATTAGCCG GCGTGGTGG
42851 CGTGCACCTA TACTCCCAGC TACTTGGGAG GCTGAGGCAG GAGAATCGCT
42901 TGAACCTGGA AGGCGGAGGT CGCAGTGAGC CGAGATCGTG CCATTGCACT
42951 TCAGCCTGGG CGACAGAGCG AGACTCTGTC TCAAAAAATA TAATAATAAC
43001 AATAACTAGC CGGGCCTGGT GGCACATGCC TGTAAGTCCA GTTACTCAGG
43051 AGGCGGAGGC ATGAGACTCA GGTGAAGTAG GGAGACAGAG GTTGACAGTA
43101 GCCAAGATCA CACCACTGCA CTCCAGCCTG GTTGACAGAG CGAGACTCTG
43151 TCTCAAAAAA AAAAAAATCC CATTGCTCA TTTTTTGGAT ACTAGTATAA
43201 CTATCACTCT AAACCACTTA GTACTTAAAT CAAGCAGATA TGGGAGATGG
43251 TGAATTACCA TCTACAGTGT TGTCATATAT GTCACATACT GAGCATTATC
43301 AGCTAGTAGA ATCTAGTTAA TTGTTCTATG TGTGATGTAT GCAGAGTTCC
43351 CATTGTTGAAT GTGTTTTTAC TATGCTTAAA TAAATGACTG ATGTCAGCAA
43401 CCCCCAAATG ATACATCTGA TGTAAGAGCC CCTGTTCCCC AATAATAACA
43451 TCTAAACTAT AGACATTGGA ATGAACAGGT GCCCCTAAGT TTCCTCCCTC
43501 CAGGGTTTCT TGGCCGGTCT CTGAGGACTA CACATCCCTA CTCCCGTCTT
43551 TCCTCATCTT CAGGCGCAGT AACAGTATCT CCAAGTCCCC TGGCCCCAGC
43601 TCCCCAAAGG AGCCCCTGCT GTTCAGCCGT GACATCAGCC GCTCAGAATC
43651 CCTTCGTTGT TCCAGCAGCT ATTCACAGCA GATCTTCCGG CCCTGTGACC
43701 TAATCCATGG GGAGGTCTCG GGGAAGGGCT TCTTTGGGCA GGCTATCAAG
43751 GTGAGCGCAG GCAACAATTG CTTTGCTCTT CTGCCCCCAG TCCCTCTGTC
43801 ACTGTCTTTC GGGGATTCT CATCACTTGG CCCCACCCCA CACCATGCAG
43851 GATGCCAGGC CTCCTTCTCG GCTTTGGGTG TTGGTGTGAG AGGTATCCTT
43901 CACCCCAACC CAGGCCACCT AAGGTCAATG TTGCTGTTAC AGTGAGCTTG
43951 TGGACCTGGA GATCCAGGTT GGGTTGAGCT GTGCCTGTGG CCCTCCTGCC
44001 TCCAGTCAGT GGGTGTGTTG TAGGTGCCTG CAGACCTCAG TACCGGGCAT
44051 GCTACAAGGA GCACACAGGG GAATGGCTCC TGCCTCCCTG GTGAACAGTC
44101 TCAGGGACTA ACCTCTCTCT TTCTCTCTC CTCCTCCTCT TCTGCTGAGA
44151 ACTGGGAGGG GGGGTCAAGT AAGACGTGTG TCTCAGCTTG GGGGCAGCAG
44201 GGCTGGAGAG CTCACCCCG ATCCACCCAG CTCCTGGTG CATGCTTTTG
44251 GCACTGACCT TCCTGCCCC AGACTTCTGT TCACTCAGGA GACTCACTTC
44301 TATGCCAAAT GACCAGAGCC CCTGCTTGGC TTGGCAGCAT CCCCTCCTGC
44351 CTTCTTCCCC ACTTCCCTTT TCTGGGTTCT TGCCTGTCCT CTGTGCATGC
44401 CCAGCTCTCC AGGAAAGAGG GTTTGCTTCC GTGTGAGTCC CATGTTGCTC
44451 CACGCTGCAT CTTCCACACA TGAAGTCTGT CATTCTGACC CGGCTCAGTG
44501 TGCCCTCCAA GGGATGGGAT GGCCAGCTGC ATAGATTTTC TCAAACAGTT
44551 CTCCAGAACT TCCTCTGGTC TCAGCACCAT TAACAGTCAC CCTCCCTGTA
44601 GGTGACACAC AAAGCCACGG GCAAAGTGAT GGTGATGAAA GAGTTAATTC
44651 GATGTGATGA GGAGACCCAG AAAACTTTTC TGACTGAGGT AAGAAGATGG
44701 AGGGGGCCCG GGAGGTTGGT GTCACCATG GAAGAGAGAA GACCTTACAA
44751 ATAATGGCTT CAAGAGAAAA TACAGTTTGG AATTACTGTC TTAAAGACTA
44801 AGCAGAAAAG AGCCCTAGAG GAATATCCCA CTCCTCTAA ATTACAGCGT
44851 AATTATTTGT TCAATGAACA CTTACTAAAA GCAACACAAA CAGGGTACAA
44901 GGGATGCAGT AACAAAAGAT ACAGGGTTCA GAAGAGCTCT CAGGTTATGA
44951 GGATGATGGA CATGAAAACA CTCCAATTGA GTACAACTCA ATGTTATAAT

FIG.3-18

45001 CCTCACCTGA ACGCCCTGCT AAGGGAGCCT GGAGGGGAGC TCCCTGAGCA
45051 CTCACACTCC TTGGGCATTT ACAGTTTTCA CTACCCCTCC CAAGTTACTT
45101 CATGGAGTAA CTTAAGTTGG GGACACCTGT GGTCTGGGTA TTGCCCTCCA
45151 AGCCACTTGG CCACTCCCAC CCCAGTTCTC CCAATGCAGT TCCAAGGGTA
45201 AGGCCTATGA AGCCATCTCC ATCTATATGG TGGTGGTCTT CCCTCATCCT
45251 GATCTTAGTG CCCTGTCATA TCACAAGATA GGAGGTAGGA GATACAGGTG
45301 GTAAACACTG TCAAGCTGAT TCCTTGGAGG GAAGAGGTAA GGAAGACAGT
45351 GAGAAGTTAA CCACCAGCTT TCCTTGGCTT CCCCCACCC CAGGTGAAAG
45401 TGATGCGCAG CCTGGACCAC CCCAATGTGC TCAAGTTCAT TGGTGTGCTG
45451 TACAAGGATA AGAAGCTGAA CCTGCTGACA GAGTACATTG AGGGGGGCAC
45501 ACTGAAGGAC TTTCTGCGCA GTATGGTGAG CACACCACCC CATAGTCTCC
45551 AGGAGCCTTG GTGGGTTGTC AGACACCTAT GCTATCACTA CCCTAGGAGC
45601 TTAAAGGGCA GAGGGGCCCT GCTTTGCCTC CAAAGGACCA TGCTGGGTGG
45651 GACTGAGCAT ACATAGGGAG GCTTCACTGG GAGACCACAT TGACCCATGG
45701 GGCCTGGACC ACGAGTGGGA CAGGGCTCAA CAGCCTCTGA AAATCATTCC
45751 CCATTCTGCA GGATCCGTTT CCCTGGCAGC AGAAGGTCAG GTTTGCCAAA
45801 GGAATCGCCT CCGGAATGGT GAGTCCCACC AACAAACCTG CCAGCAGGGC
45851 GAGAGTAGGG AGAGGTGTGA GAATTGTGGG CTTCAGTGA AGGTAGAGAC
45901 CCCTTCCTAT GCAACTTGTG TGGGCTGGGT CAGCAGCTAT TCATTGAGTT
45951 TGTCTGTGTC ACTGAACTG ACCCCAGCCA ACTGTTCTCA GTTCACAGCC
46001 CTGTTTTCAA AGAATTACAC ATCTCTAAAG GCAAACAGGG CACGGACAAG
46051 GCAAACCTGA GAGGCAAAC GTAGCCTGAG ATGGCCTGGG CTTGCCATCA
46101 CAGGTATTCA GGTGCTGAGG GCCCTAGAC CAACTAGAGC ACCTCACTGC
46151 CTAGGAAATC AATGAAGGGG AAATGAGTTC TAGCGGAGCC CTGAAGGATC
46201 AGAATTGGAT AAAGTTCTTA TTGGCAGAGA GGCACCAGGA TTGAAGTGAC
46251 AGGAGCAAAG ACCTGGGAGG AAAGAGGAGA AAATCATCTA TTTACCTGG
46301 AAACAAATGA TTCCAAGCAT AGAAATAATA ACAGCTGACA AGTACTGAGT
46351 GCCCTCTATA TGCTAGGCAC TGGGCTGAGG GATTAACATG CATGTGCATG
46401 TTTATTCCTC ATGACAACCT TGGTTTCCAG ATAAGCTGGA CTGGAAAGGG
46451 ACAGAGCTGG GATCCTGGGC TAATCAGTCT GGTGCGCAAG CCTGAGACTT
46501 TAGCCACTGC CTTTACATG GGGGTCCATG AAAATAGTAG TAGTCTGGAA
46551 CAGTTTGGGG GTACATCAAG GTCGCTGTGT TTTAAGCTAT GGAGTCTGGA
46601 CTATAGGAGA CAAATGTAAA AGAGTTTTTT GGTGACTGG CTTTTTGGTT
46651 TTTTGTGTTG TTTGTTGTTT TGTGTTGTTT TTTGTTGTTT TTTTCTGTT
46701 TCTGGGGCTT GAATCAGGAA GGAGGTTTTT TTGTTGTTGT TGTTTGTAGA
46751 AAGGATATTG CTCTGTTGCC CAGACTGGAG TGCACTGGCA CGATCATGGC
46801 TCACTACAGC TTCGACCTCC TGGGCTCAAG CAATCCTCCT GCCTTAGCCT
46851 CCCAAGTAGC TGGACTACAG GTGTGTACCA CCACACCTAA TTTTTTGAAT
46901 TTTTTTTTCT TTTTTTTTTT TTTTTTTTTT GGTAGAGACA GGTTCTCACT
46951 TTGTTGCCCA GGCCTGAATC TCAAACCTCT GGGCTCAAGC ATTCCTCCTG
47001 CCTCGCCCTC CCAAAGTGTT GGGATTACAG TTGTGAGCCA CCATGCCCGG
47051 CAGGAAAAGA TTTTAAAGCA AGAAAGCTTA AGAGCTGTGG TTTTCCAAA
47101 ATGAGTCTGG GCTGGCACAG TGGCTCATGC CTGTAATCCC AGCACTTTTT
47151 TGGGAGGCCG AGGTGAGTGG ATCACTTGAG GTCAGGAGTT TGAGACCAGC
47201 CTGGCCAACT GGTGAAACCC CTGTTTCTAC TAAAGAAAAA AATGCAAAAA
47251 TTAGCTGGGC GTGGTGGTGC ACGCCTGTAG TCCCAGCTAC TCAGGAGGCC
47301 GAGGCAGGAG AATAGCTTGA ACCTGGGAGG CAGAAGTTGC AGTGAGCCAA
47351 GATCACACCA CTGCATTCCA GCCTGGGTGA CAGAGTGAGA CTTTCTCTCA
47401 AAAAAAAAAA AAAAGAGAGA CTGATATGGT TAGTACATTG GGGTGAATG
47451 CGGAGGGTCC AGGGAATGGA GCCCTGCATA GGGGGCTAAT GAAACATTTC

FIG.3-19

47501 AGATTTCTGA ATTAAGGTAG TGGCTGTGGG GACAGGAGCC TGGGAGGCAG
47551 GGTGGAGTCA GAATGGAGAG ACTGGTTGGC AATGAGGGAA CAGGAGGAGG
47601 AGGAGGAGGA GTTACGAGTG GCTTGAGGTG TCACTTACCA GACATTTGGG
47651 GGATGGGGGA TAGCCGTGAT TGTTGAGCAA CTGGTTTGGG AAGAGCTAGC
47701 ATTGATCCCT GCTGTTCTGT GCTAGCAGAA CCTATCAGCA TCTTCTGGGC
47751 AGGAAACTGG CTCCATGAGA CTGGCTTAGG GAGAGGCTGC TAGTCACCTA
47801 ATCTGCAGAG AAGGGGCAGC TGGAGCTGTG GGACAGAAGA GGCATCCATG
47851 TAGCTGGTGG GGGTGTCTCA GCTTGTGAAG AGGAGATGGC TTTGAGCAGG
47901 GCTGACACTG AAAAGGCTGG AAGAAAAAAA CAGACACACA AGAGTCTCAG
47951 GATCAGGTAG CATAGGAAAG TTGTGGACAG TCTTTGAGGA GCACTCCCTC
48001 AGGCAGGCAG GCAGGCAGGT CATGAGCTAT AGCGATTGAG GAAGAGCTCC
48051 CTGGGTGTGT GAGCAGCTCC AGGAGCCTAA GGGATGAAAG TAGTATTGCA
48101 GGGGGCTGGA GAGCAAGGAG TGGCTCCTTC TACATTTGCA AGGGAAGGAG
48151 AAAGGAAGTT GCTCCTGAGA GTGGTAAGAG TCAGTGGTGG AGGCCTGGAG
48201 AGGAGACATA ACAAAACAAAT TTGTTGACAA ACATTTTGGT AGGAAGGGGG
48251 AGAGCTTAAA GTTTAGACAG TGGGGAAGGT GGAGTCTTAG AGGAGGTGAA
48301 TGTCTGAAAG ACAGAGCTAG CTGGAGCAAG AAGTCACTTC TCTGTTGCAG
48351 GCAGGAAGGA TCCAAAGTGG CTCAAGCCAG AGATTGGGAG AGTGGGGAGG
48401 AGGGAGCAGC CTGGATCTAA GTAAAATGGG TAGAGGTGGA GGGGGTGCTG
48451 CAACGGCCAG GGTTTTCTGA AGTTGGGGAC ATTAGGAGAG AGCTGTGAGG
48501 GCTTTGGCCA GCCACTGTGC TAGTGATTGG TGAACCAAAG GATGGGCAGG
48551 AGATGGCAGC AGGGAAGCAG AGGAAGTCCA GGCTTCCTGT TGGTATTGGG
48601 ACAAGGGAGA GGCCATAGGA GGCCCTGGCC CTGTTGTCCA GGTGGGTTTC
48651 TGAAGCTGGG TGGGCATGGC CTGGTAGGAG AGCATCTATG GCGCCCAATT
48701 CCAGATTCAG GGTCTAGTTG ATTTGCTGGC CCTGTAGCCT CAGCTCATGC
48751 TTCTGTTCCA GGCCTATTTG CACTCTATGT GCATCATCCA CCGGGATCTG
48801 AACTCGCACA ACTGCCTCAT CAAGTTGGTA TGTCCCACTG CTCTGGGCCT
48851 GGCCTCCAGG GTCCTATCCT TCCTGGCTTC CTTGTCACAA AGGAGGCTGA
48901 CTTGTCCCTT CTGGCTAGAG GGCAGAGGTG TTGCCTAGGA GCTCCTATCT
48951 TTCCCTTCCT GCTTCTTCCA ATGCCCTTCT CTGTCCTCTG GGAGCTCCGA
49001 GACACACACA GACATAATTT CACCTTCTCT CATTAGCAAC CTTTGAAATA
49051 ATTTGATTAG AAGGGACTTC AGAAGTTTGT TGAATATATG TAGAAAACCC
49101 TGTCATTTTA CCTGCTTTTG CCCCATAGTA GTCTTGTAAG ACAGTTCATT
49151 GCTGACCCCA TTTTACAGTG GTGGCACCTG AAGCCTCAGC CTGAGGCCAC
49201 CGAGCTAGTA AATTTACAGG GACCAGTTTG AGACCAGCAT TCCTCCCACT
49251 GCCCCTCAGC TGTGGTGGTT ACAATGTTGT TTGTCTTACT GACTTGCTAT
49301 CTGGCTTCCT GGGTGTCTAC CGGCTGGCCC TGGCTCTGCC CTCTAGACCC
49351 ACACCACGCA ATCTTCATTG CTTTCCCACT TGAATGCTC CACCTTGCCT
49401 AAAGAGCTTG TCTCCCCCAA GTCTCCCAT CTACTGCCCT CACCTTGCCT
49451 TTTTCTGTCT TATCCTGGTT CTAGCCACTG CCTGAAATCA TTTTAGGAAT
49501 AAGACAGGAC AGGGAAAAAC AAAAGCAACC CCCTGTCCCA CCTCTGAGTT
49551 CCACTCTCCA AGTCCCTGAG CCTCACCTCC AGGGCTCCAG TGGCTCTGCC
49601 ATGAACCCAC TGTGGGCTGG GAGTCTGCTG TGCACAGATA CCAGACCCTC
49651 AGAAACACAA ATGCCAAGTG TGCTGTGTTT TTTGTTTTGT TTTGTTTTGT
49701 TTTTATAGTG GAGTCTCATT CTGTTTCCCA GGCTGGAGTG CAGTGGTGCA
49751 ATCTTGCTT ACTGCAGCCT CTACCTCCCG GGTCTAGTG ATTGTTCTGC
49801 TTCAGCTCC CAGTAGCTAG GACTACAGGC GTGTGCCACC ACGCCAGCT
49851 AATTTTTTTT TTTTTTTTTT TGTATTTTA GTAGAGACAG GGTTTTGCCA
49901 TGTTGGCCAG GCTGGTCTTG AACTCCTGAC CTCAGGTGAT TCACCCGCCT
49951 TGGCTCCCA AAGTTCTGGG ATTACAGGTG GAAGCCACCG TGCCTGGCCT

FIG.3-20

50001 GAGTGTGTCT ATTTGATAGA GCTTTCTGCT CTGATTCTCC CTTGCTATAC
50051 ACCTTTTCTC CCCTTCTCAG TGGCTTCTCT TGCCTATGCT TCCTCCCCAG
50101 GGCCAGGTTT GAGAACATCC CCATGAAGTC CTGACCTGTC TTTTATCCTA
50151 CCAGGACAAG ACTGTGGTGG TGGCAGACTT TGGGCTGTCA CGGCTCATAG
50201 TGGAAGAGAG GAAAAGGGCC CCCATGGAGA AGGCCACCAC CAAGAAACGC
50251 ACCTTGCGCA AGAACGACCG CAAGAAGCGC TACACGGTGG TGGGAAACCC
50301 CTA CTGGATG GCCCTGAGA TGCTGAACGG TGAGTCCTGA AGCCCTGGAG
50351 GGGACACCCG CAGAGGGAGG ACAGATGCTG CCCTTGCA TC AGAGCCCTGG
50401 GAATTCCAGG GGAGGCTGT GAAGCGTAGG ACCGGATACC CAGAGCTGAG
50451 GATATTTTTC CCTTGCCAGG TGGGGCTCA CGATTTAGCT CCTGAGCTCA
50501 GGGGGCTGGG AACTGATCAG TGTCCCATCA TGGGGGATAA GGTGAGTTCT
50551 GACTGTGGCA TTTGTGCCTC AGGGATCGCT AAGAGCTCAG GCTATTGTCC
50601 CAGCTTTAGC CTTCTCTCTC CATGGTGAGA ACTGAAGTGT GGTGCCCTCT
50651 GGTGGATAAT GCTCAAACCA ACCAGAGATG CTGGTTGGGA TTCTTGAAAT
50701 CAGGGTTGTG AGGCCTCAGA AATGGTCTGA ATACAATCCA TTTTGGAGTC
50751 TGAGGCCCAG AGAAGTTCAG TGAATTGCC CTAGGAGCATAC AGCTGCCTAA
50801 TGGCAGAGGC TAGATGAACC CTAGTCTGGT TCTTTTCCAC TTTAACGTGC
50851 AGTTTCATCC TAGGCAGTGT TATGTTATAA GGGCTCTCCA AGGCAGTTCA
50901 CCTACGGCTG AGGAAGGACT ATTTTCAGGT GGTGTCTGCG CAGGACAGCC
50951 TGTGGGGTGT CCCTACAGAA CCTGTTCTAG CCCTAGTTCT TAGCTGTGGC
51001 TTAGATTGAC CCTAGACCCA GTGCAGAGCA GGTAAGGGAT GTAAACTTAA
51051 CAGTGTGCTC TCCTGTGTTT CCAAGGAAA GAGCTATGAT GAGACGGTGG
51101 ATATCTTCTC CTTTGGGATC GTTCTCTGTG AGGTGAGCTC TGGCACCAAG
51151 GCCATGCCCG AGGCAGCAGG CCTAGCAGCT CTGCCCTCCC TCGGAAGTGG
51201 GGCATCTCCT CCTAGGGATG ACTAGCTTGA CTAAAATCAA CATGGGTGTA
51251 GGGTTTTATG GTTTATAACG CATCTGCACA TCTTTGCCAC GTTCGTGTTT
51301 CATTGGTCTT AAGAGAAGGA CTGGCAGGGT TTTTTGTTT TAGATGGAGC
51351 CTCACCTCGT TGCCAGGCT GGAGTGCA GT GGCACAACT GGGCTCACTG
51401 CAACCTCTGC CTTCTGGGTT CAAGTGATTC TCCTGCCTCA GCCTCCCAAG
51451 TAGCTGGGAC TACCGGCACA CACCACCATG CCCGGCTAAT TTTTGTATTT
51501 TTAGTAGAGA CAGGGTTTCA CCATGTTGGC CAGGCTGGTC TTGAATCCG
51551 GACCTCAGGT GATCCGCCCTG CCTCAGCCTC TAAAAGTGCT GGAATTAATA
51601 GGCCTGAGCT ACCTCGCCCG GCCAGGTTTT TTTTTTTTTT TTTTAGTTG
51651 AGGAAACTGA GGCTTGGAAG AGGGCAGTGG CTTGCACATG GTCGATAAGG
51701 GGCAGATGAG ACTCAGAATT CCAGAAGGAA GGGCAAGAGA CTGTTTCATGT
51751 GGCTGTCTAG CTAGCTCTTG GGCCAAATGT AGCCCTTCTC AGTTCCTTC
51801 AAGTAGAAGT AGCCACTCTA GGAAGTGTC GCCCTGTGCC AGGTACCACG
51851 TGGACAGAGT GAGGAATCTT GGAAAGATTC CTACCTTTAG GAGTTTAGTC
51901 AGGTGACAGC ATATCTCAGC GACTCAAACA CACACACATT CAAAGCCTTC
51951 TGTAATTCCT ACAAAGTTGT GAGGGGTAGA GGAGAGGAGA GACAAGGGAT
52001 GGTTAGGATA ATGAAGGAAT GTTTTGT TTTT TTTT TTTT TTTT TTTT TTTT
52051 AGTTTCACTC TGTACCCAG GCTGGAGTGC AGAGGTGCAA TCTTGGCTCA
52101 CTGCAGCCTC CGCCTCCCAG GTTCAAGCAA TCCTCCTGCC TCAGCCTCCC
52151 AAGTAGCTGG GACTACAGGT GTGCGCCACC ACGCCTGGCT AATTTTTGTA
52201 TTTTCAGTAG AGACAGGGT TCGCCATATT GGCCAGGCTG GTCTCAAATG
52251 CCTGACCTCA GGTGATACAC CCGCTTCAGC CTCCCAAAGT GCTGAGATTA
52301 CAGGCATGAG CTACCGTGCC TGGCCATGAA GGAAGATTTG TTTTAAAAA
52351 TTGTTTTCTT TAATATTAAT TGAACACCTC TGTCAGAGC ACTGGGCTGG
52401 TGCCAGAGGG TTTCAGACAT GAATCAGATC CAGCACCTCA TAGAGCCTTA
52451 ATCTGGCACA CACACACAGC CACAAGGAGA CACAGACAAG GCAGGGTAGG

FIG.3-21

52501 ATGAGTGGAA GCTAGGAGCA GATGCTGATT TGGAACTT GGCTTCTGCA
52551 GTGAAGCCCC TTCTTAGTCC TCTTCAGTAA CCCAGCTCTC AGTGGATACA
52601 GGTCTGGATT AGTAAGATTT GGAGAGATGA TTGGGGATTG GGGAGAGCTC
52651 TCTAACCTAT TTTACCACCT CCTCTTCTGC CATTCCTCCT GTCCACATCC
52701 CCAGCATCCC TTTCCCTTGC CAAGTATCTG TGGCCTCTGT AGTCCTTTGT
52751 AAACAGCTGT CTTCTTACCC TACAGATCAT TGGGCAGGTG TATGCAGATC
52801 CTGACTGCCT TCCCCGAACA CTGGACTTTG GCCTCAACGT GAAGCTTTTC
52851 TGGGAGAAGT TTGTCCAC AGATTGTCCC CCGGCTTCT TCCCGCTGGC
52901 CGCCATCTGC TGCAGACTGG AGCCTGAGAG CAGGTTGGTA TCCTGCCTTT
52951 TTCTCCAGC TCACAGGGTC CTGGGACGTT TGCCTCTGTC TAAGGCCACC
53001 CCTGAGCCCT CTGCAAGCAC AGGGGTGAGA GAAGCCTTGA GGTCAAGAAT
53051 GTGGCTGTCA ACCCCTGAGC CATCTGACAA CACATATGTA CAGGTTGGAG
53101 AAGAGAGAGG TAAAGACATA GCAGCAAGTA ATCTGGATAG GACACAGAAA
53151 CACAGCCATT AAAAGAAAGT TAAAAGAAG GAAATTCACC CAAACATTT
53201 GAATACAGTA AGTGTATTCA TCTTTCGATA TTCCCTGTC CATATCTACA
53251 CATATACTTT TTTTATAGT AAATAGTTCT GTATTTTGCC CTGCATTTCC
53301 CTTGTGTTTA CTATCCAGTC TTCCTGTTTA TCATTTTGT CGACAACATG
53351 AAATTCTATT GAGAGACTGT CTGAACATAT TGTAAATGTAG ATGTTCAAGT
53401 TTTTCCAGTT TCTCTTTACA ATAGGTATTT AACTACAGTG AGCAGTTTTA
53451 TGCATTTAGC TAATTTCTCC TTGAGGAAG TATTTTCAA ATTACCTTTA
53501 TTCTTCTCAG GTAATAATTT CATTATTACC AAAGTTACCC TAGGTCTTTT
53551 CAAGTGTGTG GTTAAAAAAC GAGAATCTGG CTGGGCGCGA TGGCTCACAC
53601 CTGTAATCCC AGCACTTTGG GAGGCTGAGG CTGGTGGATC ACCTGAGGTC
53651 TGGAGTTCGA GACCAGCCTG GCCAACATGG TGAACCCCA TCTCTACTAA
53701 AAATACAAAA CTTAGCCAGG CATGGTGGCA GGTGCCTGTA ACCCAGCTA
53751 CTTGGGAGGC TGAGGCAGGA GAATTGCTTG AACCCAGGGG CGGAGGTTGC
53801 AGTGAGCCGA TATCACGCCA TTGCACTCCA GCCTCGGCAA CAAGAGTGAA
53851 ACTCTGTCTC AAAAATGGGG TTCTTTTCTT GCCATCAAAA ATCATGTTTC
53901 TTTTAAAAAC AAGTTCAAAC ATTACCAAAG TTTATAGCAC AGGAAATACG
53951 TCTTCTGTAA TCTCCCTTAA CCAATATATC CCTCAACATT CTCCTACCC
54001 CCAACTCCAC CCTCCCAGGA TAACCAGTTG GGACATAATC TTTATTTAAA
54051 AATGGTTTCC GGATAGAGAA AGCGCTTCGG CGGCGGCAGC CCCGGCGGCG
54101 GCCGCAGGGG ACAAAGGGCG GCGGATCGG CGGGGAGGGG GCGGGGCGCG
54151 ACCAGGCCAG GCCCGGGGCG TCCGATGCT GCAGCTGCCT CTCGGGCGCC
54201 CCCGCCGCCG CCCTCGCCGC GGAGCCGGCG AGCTAACCTG AGCCAGCCGG
54251 CGGGCGTCAC GGAGCGGGCG GCACAAGGAG GGGCCCCACG CGCGCACGTG
54301 GCCCCGGAGG CCGCCGTGGC GGACAGCGGC ACCGCGGGG GCGCGGCGTT
54351 GCGGCCCCCG GCCCGGCCCC CCAGGCCAGG CAGTGGCGGC CAAGGACCAC
54401 GCATCTACTT TCAGAGCCCC CCCCGGGGCC GCAGGAGAGG GCGCGGCTG
54451 GCGGATGAT GAGGCCCCAG TGAGGCGCCA AGGGAAGGTC ACCATCAAGT
54501 ATGACCCCAA GGAGCTACGG AAGCACCTCA ACCTAGAGGA GTGGATCTG
54551 GAGCAGCTCA CGCGCTCTA CCACTGCCAG GAAGAGGAGA TCTCAGAACT
54601 AGAGATTGAC GTGGATGAGC TCCTGGACAT GGAGAGTGAC GATGCCTGGG
54651 CTCCAGGGT CAAGGAGCTG CTGGTTGACT GTTACAAACC CACAGAGGCC
54701 TTCATCTCTG GCCTGCTGGA CAAGATCCGG GCCATGCAGA AGCTGAGCAC
54751 ACCCCAGAAG AAGTGAGGGT CCCCAGCCCA GGCGAACGGT GGCTCCCAT
54801 GGACAATCGC TACCCCCCGA CCTCGTAGCA ACAGCAATAC CGGGGGACCC
54851 TCGGCCAGG CCTGGTTCCA TGAGCAGGGC TCCTCGTGCC CCTGGCCAG
54901 GGGTCTCTTC CCCTGCCCCC TCAGTTTTCC ACTTTTGGAT TTTTTTATTG
54951 TTATTAACT GATGGGACTT TGTGTTTTTA TATTGACTCT GCGGCACGGG

FIG.3-22

55001 CCCTTTAATA AAGCGAGGTA GGGTACGCCT TTGGTGCAGC TCAAAAAAAA
55051 AAAAAAAAT GATTTCCAGC GGTCCACATT AGAGTTGAAA TTTTCTGGTG
55101 GGAGAATCTA TACCTTGTTT CTTTATAGGC CAAGGACCGC AGTCCTTCAG
55151 TAACACCAGT GTAAAAGCTT GAGGAGAAAT TGTGAAGCTA CACAGTATTT
55201 GTTTTCTAAT ACCTCTTGTC ATTCTAAATA TCTTTAATTT ATTAATAAAT
55251 ATATATATAC AGTATTGAAT GCCTACTGTG TGCTAGGTAC AGTTCTAAAC
55301 ACTTGGGTTA CAGCAGCGAA CAAAATAAAG GTGCTTACCC TCATAGAACA
55351 TAGATTCTAG CATGGTATCT ACTGTATCAT ACAGTAGATA CAATAAGTAA
55401 ACTATATTGA ATATTAGAAT GTGGCAGATG CTATGGAAAA AGAGTCAAGA
55451 CAAGTAAAGA CGATTGTTCA GGGTACCAGT TGCAATTTTA AATATGGTCG
55501 TCAGAGCAGG CCTCACTGAG GTGACATGAC ATTTAAGCAT AAACATGGAG
55551 GAGGAGGAGT AAGCCTGAGC TGTCTTAGGC TTCCGGGGCA GCCAAGCCAT
55601 TTCCGTGGCA CTAGGAGCCT GGTGTTTCCG ATTCCACCTT TGATAACTGC
55651 ATTTTCTCTA AGATATGGGA GGGAAATTTT TCTCCTATTG TTTTAAAGTA
55701 TTAACCCAG CTAGTCCAGC CTTGTTATAG TGTACCTAA TCTTTATAGC
55751 AAATATATGA GGTACCGGTA ACATTATGCC CATTTCTCAC AGAGGCACTA
55801 CTAGGTGAAG GAGTTTGCCT GACGTTATAC AACCAGGAAG TAGCTGAGCC
55851 TAGATCCCTT CCACCCACCC CATGGCCCTG CTCATGTTCC ACCTGCCTCT
55901 AATTTACCTC TTTTCTTCT AGACCAGCAT TCTCGAAATT GGAGGACTCC
55951 TTTGAGGCC TCTCCCTGTA CCTGGGGGAG CTGGGCATCC CGCTGCCTGC
56001 AGAGCTGGAG GAGTTGGACC ACACTGTGAG CATGCAGTAC GGCTGACCC
56051 GGGACTCACC TCCCTAGCCC TGGCCCAGCC CCCTGCAGGG GGGTGTCTA
56101 CAGCCAGCAT TGCCCTCTG TGCCCATTC CTGCTGTGAG CAGGGCCGTC
56151 CGGGCTTCTT GTGGATTGGC GGAATGTTTA GAAGCAGAAC AAGCCATTCC
56201 TATTACCTCC CCAGGAGGCA AGTGGGCGCA GCACCAGGGA AATGTATCTC
56251 CACAGGTTCT GGGGCCCTAGT TACTGTCTGT AAATCCAATA CTTGCCTGAA
56301 AGCTGTGAAG AAGAAAAAAA CCCCTGGCCT TTGGGCCAGG AGGAATCTGT
56351 TACTCGAATC CACCCAGGAA CTCCTGGCA GTGGATTGTG GGAGGCTCTT
56401 GCTTACACTA ATCAGCGTGA CCTGGACCTG CTGGGCAGGA TCCAGGGTG
56451 AACCTGCCTG TGAACCTCTGA AGTCACTAGT CCAGCTGGGT GCAGGAGGAC
56501 TTCAAGTGTG TGGACGAAAG AAAGACTGAT GGCTCAAAGG GTGTGAAAAA
56551 GTCAGTGATG CTCCTCCCTT CTAATCCAGA TCCTGTCTT CTTGGAGCAA
56601 GGTGAGGGA GTAGGTTTTG AAGAGTCCCT TAATATGTGG TGGACAGGC
56651 CAGGAGTTAG AGAAAGGGCT GGCTTCTGTT TACCTGCTCA CTGGCTCTAG
56701 CCAGCCAGG GACCACATCA ATGTGAGAGG AAGCCTCCAC CTCATGTTTT
56751 CAAACTTAAT ACTGGAGACT GGCTGAGAAC TTACGGACAA CATCCTTTCT
56801 GTCTGAAACA AACAGTCACA AGCACAGGAA GAGGCTGGGG GACTAGAAAG
56851 AGGCCCTGCC CTCTAGAAAG CTCAGATCTT GGCTTCTGTT ACTCATACTC
56901 GGGTGGGCTC CTTAGTCAGA TGCTTAAAC ATTTTGCCTA AAGCTCGATG
56951 GGTCTGGAG GACAGTGTGG CTTGTCACAG GCCTAGAGTC TGAGGGAGGG
57001 GAGTGGGAGT CTCAGCAATC TCTTGGTCTT GGCTTCATGG CAACCACTGC
57051 TCACCCTTCA ACATGCCTGG TTTAGGCAGC AGCTTGGGCT GGGAGAGGT
57101 GGTGGCAGAG TCTCAAAGCT GAGATGCTGA GAGAGATAGC TCCCTGAGCT
57151 GGGCCATCTG ACTTCTACCT CCCATGTTTG CTCTCCCAAC TCATTAGCTC
57201 CTGGGCAGCA TCCTCCTGAG CCACATGTGC AGGTACTGGA AAACCTCCAT
57251 CTTGGCTCCC AGAGCTCTAG GAACTCTTCA TCACAAC TAG ATTTGCCCTC
57301 TCTAAGTGTC TATGAGCTTG CACCATATTT AATAAATTGG GAATGGGTTT
57351 GGGGTATTAA TGCAATGTGT GGTGGTTGTA TTGGAGCAGG GGAATTGAT
57401 AAAGGAGAGT GGTGCTGTT AATATTATCT TATCTATTGG GTGGTATGTG
57451 AAATATTGTA CATAGACCTG ATGAGTTGTG GGACCAGATG TCATCTCTGG

FIG.3-23

57501 TCAGAGTTTA CTTGCTATAT AGACTGTACT TATGTGTGAA GTTTGCAAGC
57551 TTGCTTTAGG GCTGAGCCCT GGACTCCCAG CAGCAGCACA GTTCAGCATT
57601 GTGTGGCTGG TTGTTTCCTG GCTGTCCCA GCAAGTGTAG GAGTGGTGGG
57651 CCTGAACTGG GCCATTGATC AGACTAAATA AATTAAGCAG TTAACATAAC
57701 TGGCAATATG GAGAGTGAAA ACATGATTGG CTCAGGGACA TAAATGTAGA
57751 GGGTCTGCTA GCCACCTTCT GGCTAGCCC ACACAACTC CCCATAGCAG
57801 AGAGTTTTCA TGCACCCAAG TCTAAAACCC TCAAGCAGAC ACCCATCTGC
57851 TCTAGAGAAT ATGTACATCC CACCTGAGGC AGCCCCTTCC TTGCAGCAGG
57901 TGTGACTGAC TATGACCTTT TCCTGGCCTG GCTCTCACAT GCCAGCTGAG
57951 TCATTCTTA GGAGCCCTAC CCTTTCATCC TCTCTATATG AATACTTCCA
58001 TAGCCTGGGT ATCCTGGCTT GCTTTCCTCA GTGCTGGGTG CCACCTTTGC
58051 AATGGGAAGA AATGAATGCA AGTCACCCCA CCCCTTGTGT TTCCTTACAA
58101 GTGCTTGAGA GGAGAAGACC AGTTTCTTCT TGCTTCTGCA TGTGGGGGAT
58151 GTCGTAGAAG AGTGACCATT GGAAGGACA ATGCTATCTG GTTAGTGGGG
58201 CCTTGGGCAC AATATAAATC TGTAACCCA AAGGTGTTTT CTCCCAGGCA
58251 CTCTCAAAGC TTGAAGAATC CAACTTAAGG ACAGAATATG GTTCCCGAAA
58301 AAAACTGATG ATCTGGAGTA CGCATTGCTG GCAGAACCAAG AGAGCAATGG
58351 CTGGGCATGG GCAGAGGTCA TCTGGGTGTT CCTGAGGCTG ATAACCTGTG
58401 GCTGAAATCC CTTGCTAAAA GTCCAGGAGA CACTCCTGTT GGTATCTTTT
58451 CTTCTGGAGT CATAGTAGTC ACCTTGCAGG GAACTTCCTC AGCCCAGGGC
58501 TGCTGCAGGC AGCCCAGTGA CCCTTCCTCC TCTGCAGTTA TTCCCCCTTT
58551 GGCTGCTGCA GCACCACCCC CGTCACCCAC CACCCAACCC CTGCCGCACT
58601 CCAGCCTTTA ACAAGGGCTG TCTAGATATT CATTTTAACT ACCTCCACCT
58651 TGGAAACAAT TGCTGAAGGG GAGAGGATTT GCAATGACCA ACCACCTTGT
58701 TGGGACGCCT GCACACCTGT CTTTCCTGCT TCAACCTGAA AGATTCTGTA
58751 TGATGATAAT CTGGACACAG AAGCCGGGCA CGGTGGCTCT AGCCTGTAAT
58801 CTCAGCACTT TGGGAGGCCT CAGCAGGTGG ATCACCTGAG ATCAAGAGTT
58851 TGAGAACAGC CTGACCAACA TGGTGAACC CCGTCTCTAC TAAAAATACA
58901 AAAATTAGCC AGGTGTGGTG GCACATACCT GTAATCCCAG CTAATCTGGA
58951 GGCTGAGGCA GGAGAATCGC TTGAACCCAC AAGGCAGAGG TTGCAGTGAG
59001 GCGAGATCAT GCCATTGCAC TCCAGCCTGT GCAACAAGAG CCAAACCTCA
59051 TCTCAAAAAA AAAAA (SEQ ID NO:3)

FEATURES:

Start: 3000
Exon: 3000-3044
Intron: 3045-45393
Exon: 45394-45525
Intron: 45526-45761
Exon: 45762-45818
Intron: 45819-50154
Exon: 50155-50329
Intron: 50330-51076
Exon: 51077-51132
Intron: 51133-52775
Exon: 52776-52933
Intron: 52934-55922
Exon: 55923-56064
Stop: 56065

FIG.3-24

CHROMOSOME MAP POSITION:
Chromosome 22

ALLELIC VARIANTS (SNPs):

DNA Position	Major	Minor	Domain
941	A	T	Beyond ORF(5')
2612	G	A	Beyond ORF(5')
5080	G	A	Intron
6599	-	A C	Intron
6983	C	G	Intron
9885	A	-	Intron
12538	G	T	Intron
17707	T	C	Intron
18219	-	A	Intron
19670	C	T	Intron
21153	G	T	Intron
24566	C	-	Intron
26604	G	A	Intron
27255	C	G	Intron
27399	T	C	Intron
28088	G	A	Intron
28734	G	A	Intron
29246	-	T	Intron
29490	G	A	Intron
29934	T	C	Intron
34480	A	G	Intron
38812	T	C	Intron
40731	C	G	Intron
41303	T	A	Intron
41305	-	A	Intron
41457	G	C	Intron
43168	A	- T	Intron
43357	T	G	Intron
45664	T	C	Intron
47549	A	C	Intron
47908	C	A	Intron
52267	C	A	Intron
54654	T	C	Intron
54679	C	G	Intron
54693	A	C	Intron
54706	T	C	Intron
54712	T	C	Intron
54799	T	C	Intron
54819	G	A	Intron
55499	C	T	Intron
56825	C	A	Beyond ORF(3')
58871	T	A	Beyond ORF(3')

Context:

FIG.3-25

DNA
Position
941

GAGTAAGTGGGTGGTCAGGTTACAGACTTAATTTTGGGTAAAAAGTAAAAACAAGAAAC
AAGGTGTGGCTCTAAAATAATGAGATGTGCTGGGGGTGGGGCATGGCAGCTCATAAACTG
ACCGTGAAAGCTCTTACATGTAAGAGTTCAAAAATATTTCCAAAACCTTGAAGATTCT
TTGGATGTTTGTGTTTCAATAAAATCTCTCACTAATTCATTGTCTTGCCACTGTCCGTAA
CCCAACCTGGGATTGGTTTGAGTGAGTCTCTCAGACTTTCTGCCTTGAGTTTGTGAGAG
[A,T]
GATGGCATACTCTGTGACCACTGTCAACCCTAAAACAAAAAGGCCCTCTTGACAAGGAG
TCTGAGGATTTTAGACCCAGGAAGAATGAGTGATGGGCATATATATATCTATTACTGAG
GCATGAGAAGAGTGAATGGGTGGGTGAGGTGGTGTTTAAGGCCTCTTGCCAGCTTGT
TTAACTCTTCTCGGGGAACGAGGGGGACAACGTGTGTACATTGGCTGCTCCAGAATGATG
TTGAGCAATCTGAAGTGCCAGGAGCTGTGCTTTGTCTATTTCATGGCCCCGTGCGCTGTG

2612

TGAGTTGGAACAGTTTGATACCAAAACCATCCCCCGCCCCCAACCCCGAGCCTAGGGT
CCGTGGAAAAATTGGCCCCTGGTGCCAAAAAGGTTGAGGACTGCTGATCTAGAGGACCAA
TTTATTCAATGTTGGTTGAGTAAATGAGCTCTTGATTAGGTGATGGAATACTGAAAA
AACAGGGCTTTTGAGGAATAGGAAAAGGCAGTAACATGTTTAACCCAGAGAGAAGTTTCT
GGCTGTTGGCTGGGAATAGTCATAGGAAGGGCTGACACTGAAAAGAAGGAGATTGTGTTT
[G,A]
TTTCTTCTCTCAGAGCTATAAGCAAAGGCTGAAAGTTCTAGAAAAAGGCAAGTTTGT
TCAGTAGAAAAAGGATAATCAGAACCATTTTAGAAAATGGAATGAGACTACTTTTGAG
GCCATGAGTTCCCTTGTCCTGGAGAGATGAGCAGAGGTTGGACAAGTGCTTACCAGAGAT
CTTGTTGGAGGCAGAACTGTGCATCTAGCAGAGCATTGGCCTAACCTTTCAAATGAGAT
GCTGTTAACTCAGTCTTATTCTACATGGTAGGAATCCTGTCCCTTTGCTCCTGCTACTT

5080

ACAACGTAAATAGTTGAAATTTGTTGGTGGAAAGAAGAGCAGTCCACTCCAGAGGCTGG
ATGGGCATGCCTGGCCCCAAGGTCTGAAGTGGTAGGGCTGTGCCTATATCTGAGAATG
AGATAGACTAGGCAGGCACCTTGTGCTGTAGATTCCAGCTCCTGCACATAGCTCTTGTTG
TAAACATCCCTGTGCTTATACCAAGTAATTGAGTTGACCTTTAAACACTTGCCTCTTCC
CTGGGAACCATATAGGGGATTGGCTGGAGACGTCTGGCCTCTGGAAGAGTTGGAAGCA
[G,A]
CCATCATTATTATCCTTTCTTTTCACTATAACTCAGAGCTCTCAAGTCTTTTCTGTGGA
TCTTATTGCCTTGGTTCTTGCCCTTTTACTCCAGGGAAGTTGATTCTGTCTTTTCTGT
TCCATTTAGTATGACAGGAGCAGAGAATGTCAGAGCTGTAAGGGACCTTATAGTTAAAGC
CTTTGGCTGGTCTTTTCAATTTATAGCTGGGACTAATAAGTAACGTCAAACCCAATGAG
TTCACAGATTGGGTCTCGCCTTGGCATGTAACCCATATGTTTATATTCTTGCTGTTTTCC

6599

CTGTAATCCTAGCACTCTGGGAGGCCGAGGCAGAAGGATCGCTTGAGCCCATGAGCCCAG
GAGTTTGAGACCAGCCTGGCCAACATGGCAAACTCCACCTCTACAAAAATACAAAAAT
ATTAGCCAGGCGTGATGGCACACACCTGTAGTCCAGCTACTTGGGAAGCTGAGGAGCGA
TGATTACCTGAGCCCAGGGATATCAAGGCTGTAGTGAGCTGTGATCATGCCACTGTACTC
CATCCAGCTGGGGACAGAGTGAAACCCCTGTCTCAAAACAAAACAAATGAAAAAAAAA
[-,A,C]
CCTTAATAATCAGTAACTGTCACTTTATATTATGTTGTGAGTGTGTGTCTATATACACCT
ATATGTATACATTTCTCTTATTACACATTCATTGGTGATCTGATGTGGAGCCCCAGGGAT
TAAGGGCAACTTTGAACTACCCTGACACAATCAAGCCAAATATCATTCCCGTGGAGGAAG
TAGAGTATCTAGGTTCTGTCTCCTAGTTGCAGCTTTACCTTGAGGACAGAGACTCTAATC
CAGCTGTGCTGAAGGAGCACATCTCCTGACTTCTGAGCTTTCCCTGGTAAATTCAAACT

FIG.3-26

6983

CACATTCATTGGTGATCTGATGTGGAGCCCCAGGGATTAAGGGCAACTTTGAACTACCCCT
GACACAATCAAGCCAAATATCATTCCCGTGGAGGAAGTAGAGTATCTAGGTTCTGTCTCC
TAGTTGCAGCTTTACCTTGAGGACAGAGACTCTAATCCAGCTGTGCTGAAGGAGCACATC
TCCTGACTTCTGAGCTTTCCCTGGTAAATTCAAACCTGGATGTCACGGCGCCCTCAGATA
GAGCCTGGTAATTTGCCCTGGGGAGAGTGACTGTCTTTTGATCTAATTTGACTTTTGCC
[C, G]
CAGTTGGAGGAAAATCTTCAGGGCTAGGAAGGATTGTATTTGTCTGACCCCAGAGATAAC
CTGGGTTTTGAGGAACATGGGGCATCAACCTGAATGGTCTTGTAAGATCTCTCCACGCC
AGCTTGCCAGTGTTTTCTGATGAATTTAGAGTACCTGAGTAGTGCAGGCCTGCTGGGAG
GAGGACTCTCCCTCTGTGCTACTCAGAGAAATTCATTCTTCAAGGCCCTTCCAGCCTT
GCTCTTACCCAGCTGGGCTACAGTTACAATAAAGGAAATGACTTTTCTTCTCCCTTCC

9885

GGCGTGCCACCACACCTTGCCATTTTTTTTTATTTAAGTAGAAAACAAGGTCTTATTAAT
ACTATGTTGCCAGGCTGGTCTTGAATCCAGCGATCCTCTGCCCCAGCCTCCCAAAGT
GCTTGGGATTACGGAAGTAAGCCACTGTGCTGGCCAGTGCAACCCCATTTTATACTAA
AACAGGAAGGCCAGAAAAGGTTTGGAGTAACCTGTCCAGGGTCACACAGATGATTTGA
ACTCAGGTCTCCCTGGCTCCCAAGAGAGTCTGCTTTCCACTAGGACTCCAGGAGAAAAA
[A, -]
AAAAAAAAAACAGTAGACTTGAGACAGAAAATCTGATTTGAGTCTTAGTTGAGCTAGG
CTAACTGTGTAAGTGTGGGCAAGTTCCTTAGCCCTGTGAGCCTCAGTTTCTTATCTGTA
AAATGTCATAAAAGAAATCCATCTCATGGAGTAGTTGTGATGATCAAGGACTCTGAAAAC
ATTAGAATGGTTTAAATGTGAAGGATTAGCAGCAGCATGGCAACATTGTGCATCTTATA
TTAACTATCCAAATATATCAAGCGTCATTTGCTATATATAAAAGTCATCAAATTAGGCAC

12538

ACTTGGGAGGCTGAGGCAGGAGAATCACTTGAACCTGGGAGGCAGAGGTTGCAGTGAGCC
CAGATCACGCCACTGCACTCCAGCCTGGTGACAGAGTAAGACTCCATCTCAAAAAAAAAA
AAAAAAAAAAAAAATTCCTTAATTTGGCCTACAGTAGAGCCCTCCGTAATGTGGCCTCTCT
CCACATCTCCACAACCTCCTGCTCCCTGCACTTCAGCCTCACCTCTCTTCTGGACAGGCC
CTCCTTCTGACAAGGGCTTTGTTTATTCTGCTCCCTCTGCCTAGAATGCCCCCTTACTCT
[G, T]
TTCACCTAACTCCTGCTTATCGTTTAGATCTTTACCTGGATGGCTCAGAGAAATATAGAA
GTAATTCCTCACCTGAAAAATAGGTTAGGTCCCTGTTTTATGTTTTCATAGACCTTTCC
TTTGAGGCTTTTTTAAAAAGTAGTTTAAATCTCACATTTATTCATGTGATCATCTCCT
TAATGATATCTTAAGACCTCTAATAGAACAATTTGGTCATGGACTGTGGGGTTTTTGCCC
CTCATTGTGTCAGCACTGAGCATATTGTTGGCATAGGAGGGATATTGTTGAATGAATTG

17707

GTAGTGGTGCTCAGAGTGTGCTGGGTGAATGATGTATTTGTTGAACGACTCTTGGA
CACTTGAATAAAGTCCATCCAGTATGCACCATTACCATCTCTTCGCTCTACAATATTCTT
TTAGGCAAGAGCTTATCTTTTGAGGTGATAAGATAAGCTCAAACCTATGTAGACTAAGAC
CTCAGTCTGTAAATGTCATCCCTAAGTCTTAAACCATCAAACAGGGCCTCAAGGAATG
GCATGCCTTCTGCAACTGTAGCAACCTGCTGTGCTTATTTGCCGTGTTTTTCATTTTTT
[T, C]
CCCAAAAGCTAGAGTCCCTTCTCCCATGGGCAGTGCTGGAAGTGTGCTAACAAATCTTT
CTCCATACTGCTTACGATTACAAAAAAACCTCAGCATCTCATGCCAGACTTGAGTTAA
GGTTGTTTTCTTTGTGTGTCAGCTGTATTCTGGTCATGACTTCTGATGATGCCCTATA
GAGATTTTGCTGAGATCAGAGGGTGCTCCACTGCCATCAGTAGCACTGACTCTTGAGAA
GCACCGTTTCTGAAGTTGGCTAATGTCATCCCTCACGTTTGTGTTGAAATTTGTTTT

FIG.3-27

18219 TGCCATCAGTAGCACTGACTCTTGCGAGAAGCACCGTTTCTGAAGTTGGCTAATGTCATCC
CTCACGTTTGTGTTGTTGAAATTTGTTTGTAGTTCCAGAGATAGCACTTTCATGGAATGAC
GCTATCTTCTAGAATCACTTTTTTTTTTTTTTTGAGTTGGAGTCTCGCTGTGTGCCAGG
CTGGAGTGCAGTGGCACAATCTCAGCTCACTGCAATCTCCACCTTCCGGGTTCAAGTGAT
TCCCCTGCCTCAGCCTCCCAGGAGCTGTTACTACAGGCGCACACCCCCACTCCTGGCTA
[-,A]
TTTTATGTGTTTTAGTAGAGACGGGGTTTACCCTGTTGGCCAGGATGGTCTCGATCTCC
TGACTTTGTGATCTGCCTGCTTACGCTCCCAAAGTGCTGGGATTACAGGTGTGAGTCAC
CGCGCTGGCCTAGAATCACTTTTTATACCATAACGTGAGCACCCTGCCGCGTCACCA
AGGAAAGAGAGAGGCAGCTACTGTGGGGTTACAAATGGGTAAGAGTGGCACCAGGAAGGT
GAAAGTCTCTACTTAGCCAAGGCTTAACAAATGTCAATCACCACCAATTTATTTATTA

19670 GACCCCATGATGAGCAACTATAGCACTAGAACAGTGATAATAACTAATGTTTATAATGC
ATCTTCAGTTTACAGAGGGCTTTTGTACTCATCATCTAGTTTAGTTCCGCAACAACCTC
TTGAGGAATATAGCACAAGCAGGACAAGGGAAGCCAGAGATGTTAAATAATTTATCCAA
GTTTATGCTGCTGGGAAGGGCAGCACTGAAATTAAGAAAGTTTCTGAGCTCAAATC
CCATGCCCTTTCCTCAATGTGAGCTCTAGCAAGGTATTAGGAATCCTGCCTCTACAGTT
[C,T]
AGAGCCTCAAATTGCTGGGTATGTTGAGTTCTTGATCTGATTTTTCTAGATTTCTGCC
CACATTCTTACTGTCTGGATATCAGGAAAGAGTTTATCAAATGCCTGTGGAAATCCAAGA
TAAGGTCTCATGATGAGTAACCCAGTGAAACATGAAGTCAAGTCTAACTAGTCACTACT
ATTTCACTACTGCTGACTCCTGATGATCAGCTCCTTTTCTAAGTGCTTACTGTCCACTTA
TTCCATCATCTGCCTAGAATTTATGTGAAGGAATCAAAGCAAAGGATCATAAGGCTTCC

21153 GGACCCCTGTTTTAGAAGGATGACTGCTGCTATAATGTAGAAAGTGATTTGGAAGAGGGG
AGGAGTGGGGCAGGAAAGATGGTTAGTAGATGGGGTGGTAATGCTTACCTTTCAGTATT
TGGAGGCTTCGGAGTCTCAAAAATCTCTTCCTTGATTGGAGTCTCCAGCCAATAGA
GGGCTTCACACAAACAGTTTCTTGGGTTTTGAATTGTTTGACCAGAGCTTTCTCCGACA
AAAGGTTGGGGTGATTCATTCACTTACCACACCTTGCTGAACATTCACTTGGGGCTGCC
[G,T]
GTTATGAAGGCTATTGTTCTCCAGCCTGTCACAGACGCTTTGAAGACCTGTGCCTCAGCT
GGTTCTAAGGAGTCAGTTTGTTCAGCTCCGTGCCAGGTTTCCAACCTTATGAAATGTGCTG
GAGATTAACACCTCTCCTGCCATTTATCCCTACTATAATTGCCAGTCAAAGGATTCTG
CAGTTGCCCTCTGGCAGCCATAACTGATGAATGTTCTGCCAGCTGCTCTGAGGACCTAGAA
GAGCAGTTTTCTATCCAGGACAGTTTCCAAGGGTGGGAGGGTGAAATATATCCTCCAGT

24566 CTA CTCTGGAGGCTGAGGTGAGAGGATCACTTGAGTCCAGAAGGTCGAGGTCAAGATTGT
AGTGAGCCATGATGGCATCACCGCACTCCAGCCTGAGTGACAGAGAGAGACCCTGACTCA
AAAAAAAAAAAAACAAAAAAAAAAAAACACCCTCACCACCTTATCAGCTATTTGTCTTGAGAA
TAGTGACATAACCCCTCAGAACCTATTTCCCTAATCTGTTAAATGAGGCTGATGACGTTTC
CTCCTTTTACTGGCAATTTAAACATGATGGATAATAAATGCTAAGCACTTAACACAGGGC
[C,-]
TAGAAGATATTAAGTCTCAATAAATGGTAGCTTCTTAACAGTATTCAAACCCATGTGCT
CTTATCACATGCATTGTTGTCCCTGTGTCCAGTTGGTGGAAATGGGAAAAGGCTCCCTGT
AACCCCATCTACCATCTTTATCAGACTTTCCTGCCATGGTTCACAGTAAGAGATAGAAGC
TGCACGGTGACTTCTGGCTCTTTACAATGGTGAGCGGTGTGTGCCTGGTAAGGGAGAGCT
GATGTCACTGCCCCAAATCCAGTAGTGAGATCTGAGTGTCTGGTTTCTCCAGCAGCCT

FIG.3-28

26604 GATTTGCAGCTGAGCCTGTCTATCTGGTGTGGGAAGAAGATGGGGAGTTACTTGTCACTC
CCGGCTTACTTCACCTCCAGAGACCTGTTTCGGTGAGTTGGTCTCCGAGTTCCCTCTCC
ATCTCTCCTGGCCCCTGGTCTGAGAGGAGGGTGGTCTCCCTAAATCTCCTTCTCACTTA
GTCCTTTACCATCGGTTCTGCCGGGCAGAAGCCAGCGGAGGTTATACCCAAGGAGAATCG
GCCTTGTGAGGTACCCCCATTATGTCCTGGAAGTGGTGAGGGGAGGGATATACCCAGAAG
[G.A]
AACTTCTTAGGGAGCTCCAGCTCCCTTCTATCCCAGACAAACCTGAAGGAGCCTCCAAA
AGATGCCACTGACCTGCCATTGTAGATGTTACTGCTTCCGGGGGAATAGCCCAAATAG
AGTGCTGTTTCCAGCTCTCACATGTCTTACCTGCGGGCCATGCTGCCTGCCCAGGAATTT
GTCCCAACAAGCAGGATGGGCAGGTTTTGCCAACTGTGGAACTGGCAAGTCTGGGTG
TGGGTAGCCTGGTACACAGTAGGCACCTTATAAACGTTTGTCTCTTAATGGCAGGCACA

27255 TGGGGAAGACCTGGGCGAGTGCTTCTAAGACTGGAGCAATGGGCTTTAGAGTGTTCTTG
AGCTGCTGGGCCAGCCCCACACCTCCTCAGTCCCTAGGCCTAAGTACCTCCACGAGCCT
CTCTCTGTGGGGCTTCTCAGAGGGAGATGTGGAACTCTACCTCTAACCTGGCTTTCTTT
GCTCATTGCCCCACTCCACCTCCCATAGAACTCCCAAGGGGGTTTCTGGCCCTCTGGGT
CCCTTCTGAATGGAGCCATTCCAGGCTAGGGTGGGGTTTGTTCATTCTTTGGGAGCAG
[C.G]
CTGTTGTTCCAAAAGGCTGCCTCCCCCTCACCAGTGGTCTGGTGCACTTTTCCCTTCT
GGCTTCTCTAAGCTAGGTCCAGTGCCAGATCTTGCTGCCGGGATACTAGTCAGGTGGCC
AGGCCCTGGGCAGAAAAGCAGTGTACCATGTGGTTTTGTGGAATGACCGGACCCTGGTAG
ATTGCTGGGAAGTGTCTGGACAGGGGGAAGGGGAAGGGAACGGTCTCAATGCTGACT
CTACCAAGCGCCCTGCTAGACACTTTATCCTTTAATCTCTCAACAGCCTAAAGAGATTAT

27399 AGATGTGGAACTCTACCTCTAACCTGGCTTTCTTTGCTCATTGCCCCACTCCACCTCCC
ATAGAACTCCCCAGGGGGTTTCTGGCCCTCTGGGTCCCTTCTGAATGGAGCCATTCCAG
GCTAGGGTGGGGTTTGTTCATTCTTTGGGAGCAGCCTGTTGTTCCAAAAGGCTGCCT
CCCCCTCACCAGTGGTCTGGTCGACTTTTCCCTTCTGGCTTCTCTAAGCTAGGTCCAGT
GCCCAGATCTTGCTGCCGGGATACTAGTCAGGTGGCCAGGCCCTGGGCAGAAAAGCAGTG
[T.C]
ACCATGTGGTTTTGTGGAATGACCGGACCCTGGTAGATTGCTGGGAAGTGTCTGGACAGG
GGGAAGGGGAAGGGAACGGTCTCAATGCTGACTCTACCAAGCGCCCTGCTAGACACT
TTATCCTTTAATCTCTCAACAGCCTAAAGAGATTATATATCCCCATTTTACAGATGAGGC
AACCAGTTTCAACAGAGTTAACATATGGAGCCTCACTGGGCAGCTTTTTCTGTCTTCTG
ACTTCTCTCATCCTTCAGGGGCTGCAGGTTTGTTCCTTCTCTAGTGGAGAGGAAAT

28088 AAGAGCCAATGGAAATTGATCTTGAGTTTAGGAGAAAGCTTTTACATGTGGAATTAAGAT
GCCAAGTGTTGAAGTAGCCACATTTAGGTCTCATTAAATTTCTCTTAATCCTGGGAAGG
CAGCTTAGGAGAAGGGTTGTTCTTTAGGAGCCAGGAACATAACCCCTTTTACCTTGGA
GAGGCAGGGAAGCCAGGGAGGACACAACCTTCTCAGGAAGAGGAGAAGCTAGAGCAGATAG
TGAACCTCTCAACCTGAACCTTAAGGGCCAGACCACTAATGCCACCCAAGTCCACCTGCC
[G.A]
TTTGTCTTGTCTGTCCCAGGCTTTCTGGAGAACCTGATCTTCTTGCCCTACCCCCAAG
CTCCGTTTGCCAGCTAGAGTCTGGGGGTACTGACTGACTTTTCTGAGACATTCTTCCCT
TCCCCAAATAAGAGGCCACATTCCTGAAGTCACTTCTGAAGAGATAGCTGCCACACAGGG
CTCTTTCCCCCAGGGAGGGACACCCAGACCCTCTGCTCTCCAGGTATCCGTTACCAC
ATCACTACCTGGTCAGAAAGCTGTTTCTGCCATTAGCCCTCCCTCTTTTATTATAGGAT

FIG.3-29

28734 AAGTAGAAGCTAGACTTCTTGGGCTCCTGAACAGGGTCCTTGCTGGATTCTGTGAAACAA
ATTAAGTTCTTGACCCTAGGCCTCTGGGGGAGTACAAAGTCTATGGGAGTTCTGGGGCTG
TGGTTGCAAGGAAAGTGACGCAACCAGATTCCATGGGGACATGATCAGGCGTGACATGTG
AGGGAGGAAGAGGGAGCAAGGGAATGAAGAATACAACCTTCTGTGTCCCATACACCCCTGC
CTGACAGGCCATACATACTCAGCAGAGAATGCACTGTCTTCTACCACACTAGCGTGAG
[G,A]
AGTGAGCTGCAATTACCACTGTGCTTCCAAGTAAGAAAATACCTCAAATTGGAATTTACA
AAAGAGGTAAATTAGGGAGTGGCTTTTGTGGGACATCTTTAAAGCATTTTTCTTTTTATA
GAATTTCACTTAATGTCCAATACTGATTTAATGAGCTTGGGTTTACACATTATCTCTTGA
AGAAAACAAATGAACCTTTGTGTTCAAAGCAATCCATGTTTAAAGGGAAAAAATTATGC
ATAACTCTGCCAGCTTCACAGTAACCTTTGGCAGGTGCCCTTAGGTCTCTGGGACTCTT

29246 AATCCATGTTTAAAGGGAAAAAATTATGCATAACTCTGCCAGCTTCACAGTAACCTTTG
GCAGGTGCCCTTAGGTCTCTGGGACTCTTTTCTTATCTGAAAAATGAAGGACTTGGATC
AGGTGAATGGTTCCAGCTCTGCAACTTATGTGGCTCCTCAGAGGCACACAAGCTCTTTT
CCATTATTTGCCAATAATGGAGGCCCTGTCTTTAACTGCAGTACAACACACAAAATAC
TTGAAACTACAGTCTTCTGGTTTTTGGTTGGAAGTGAATCAGTGCCTCTAGCAACACT
[-.T]
ATTTCTTGCTGTTCTGAGGCTTCATTATGTGTTTGGTTAATTTTTTAAAAACAACAATAAC
ATATTCCATAATAATTACAGCTTAATTGGCAGACTGTTTCAGTCTATAGGATCTGCAGGA
AGGAGGAGTAATAAAGGGATTTTTGACTGAGCTCTTATGGAACAGAGTCTCTCTAGGCCC
CTGTCAATCTGCCCTTCTGGGCCCTGGGGAAAAGTTGGCATCCCCAGTTGTGGTGCTCT
CCAGGTGCCCTCAGGCTGTGGTGGAGGGAGCTTCCATTCTCTCCTTCAGCCCACTCAAT

29490 AACTACAGTCTTCTGGTTTTTGGTTGGAAGTGAATCAGTGCCTCTAGCAACACTTATT
TCTTGCTGTTCTGAGGCTTCATTATGTGTTTGGTTAATTTTTTAAAAACAACAATAACATA
TTCCATAATAATTACAGCTTAATTGGCAGACTGTTTCAGTCTATAGGATCTGCAGGAAGG
AGGAGTAATAAAGGGATTTTTGACTGAGCTCTTATGGAACAGAGTCTCTCTAGGCCCCTG
TCATATCTGCCCTTCTGGGCCCTGGGGAAAAGTTGGCATCCCCAGTTGTGGTGCTCTCCA
[G,A]
GTGCCCTCAGGCTGTGGTGGAGGGAGCTTCCATTCTCTCCTTCAGCCCACTCAATTCAG
AGGCTAGGGGCTGAAAGAAGCTTCTTACAACCTGGCTGTTCACTGGGAGGTAAAGGGATG
ACCATCCAGCCAGGCCCTCCTCAGGACATGGGAGGGCTTATGCTTTAACATGTGTAAATC
CACTGCAATAATGACTGGTCTTTTTACCCATAAGGTTGAGAATTTACCTGTAAACATTT
TTGTCTGAAGAATTTGGATGTAAGTGAGGGCTGGGCCCTCTATCTTATCTCACTTGGCTC

29934 GGACATGGGAGGGCTTATGCTTTAACATGTGTAAATCCACTGCAATAATGACTGGTTCTT
TTACCCCATAGGTTGAGAATTTACCTGTAAACATTTTTGTCTGAAGAATTTGGATGTAA
GTGAGGGCTGGGCCTCTATCTTATCTCACTTGGCTTCTCTCAGCACAGCACCTTGCCTGC
TTGTTCTTACACATCCTAGATGCACAGTAACTATTCTTAATTATTAGAAATCTATTAGA
ATCAATTGATTTCACTGGGCTTGGTGGCTCCTTCTGTAATCCAGCACTTTGGGAGGC
[T,C]
AAGGCTGGAGGATCACCTGAGTCCAGGAGTTTAAAGACCAGCCTGGGCAACATAGGGAGAC
CCTGTCTCTACAAAAAATAAAAAATTAGCCAGGCATGGTGGTGTGCACCTGTAGTCCCAG
CTACTCAGGAGGCTGAGGCAGGAGGATCTTTGAGCCTGGGAGGTGAGCTACAGTGAGC
AATGATTGTGCCACTGCACTCCAGCCTGGGTGACAGAGTAAGACTCTGTCTCTTAAAAAA
AAAAAAAAAAAAAGTTGATTTCTATTTGGATAGATAAATAATTCATTTTAGGACCTTTCTT

FIG.3-30

34480 CTGACTTCAAGTGATCCACCCGCTCGGCCTCCCAAAGTGCTGGGATTATAAGCATAAGC
CACTGTGCCAGCTGCTCTCTATATTTTTAATACATATTATTTCCATTAATTTTCACAGC
AGTTCATTTTATAGATGAGGAACTAGGCCAGAGAAGTAAATATCTTGCCCAAGATGAT
GTAAGTAGTAAGTGGCAGGATCAAGATTCAAACCAAGCAATGTTCAAACCTCTTGGAAGC
AAGAATGTGCCACTGTGGAAGGTGCAAGGCTTGACAACAAGAATAGGGAAAAGAAGGA
[A.G]
CTAGAAGGAAAGAGATGGCATGGGCTCAGCAGGCCAGGGAGCTCTTAGCTGTGTGTGTG
GGAAGCTCAGAAGGGAGGAAGAGGTTGTCTGTGCAGGTAAGTCTGAGAACACACCAGAC
TTTTGAGAGGTGGAGCTTCATAGCCAGGTCATTAGGGGAGAAGGGAGCTATAGATTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTAGAGACGGGGTCTTACTATGTTGCCCAGGCTG
GTCTTGAAGTCTGGGCTCAAGTGATCTCCACCTCAGCTCCCAAAGTGCTGGGATTA

38812 AAATCCAGCAGATCCATTGAGAGTTTAAGCAGCAAGGTGTTGTGACCAAGTTAACATTTT
AGAAGGATCACTGGTATGGAGGTTGGATTGGAGAGGGGAAAGCCTAAAGGTATAGAGACT
AGTTAGGAAGCTATTGTAGGCTGGGCATGGTGGTTCATGCTGTAACTCAGCACTTTGG
GAGGCTGAGGTGGGAGGATTGCTTGAGGCCAGGAGTTGAAGACCAACCTGGCCAACATAG
CAAGACCCCGTCTCTGTTTTCTTAATTAAGAAAGTCCAGACGTAGACATAGTGGCT
[T.C]
ACGCTGTAAATGCCAGCACTTTGGGAGGCCAAGGTGGGCAGATTGCTTGAGGTCAAGAGT
TTGGGATTAGGCCAGGCCAGTGGCTCAGCCTGTAACTCCAGCACTTTGGGAGGCCGAG
GTGGGCGGATCACAAGGTCAAGGATCAAGACCATCCTGGCTAACACAATGAAACCCCGT
CTCTACTAAAGTACAAAAATTAGCCGGGCATGGTGGCGGACGCTGTAGTCCAGCTAC
TCGGGAGGCTGAGGCAGGAGAATGGCGTGAACCTAGGAGCGGAGCTTGTGTGAGCAGA

40731 GTTCTGTCTATGTCTGTCTCTCGGATGAAGCTGAGCTGGCTTTCAGAAGCCTGCAGAGT
TAGGAAAGGAACCACTGGCCAGGGACAGACTATGAGGATTGTCTGACCCAGCTGCCCC
TGTGGGGATCACAGTTTACAGCCAGAGCCTGTGCGGACCCAGCTGTCTGCCAGGTTTCT
TAGAAACCTGAGAGTCAGTCTCTGTCCACTGAACCTTAAGCTGGACAGGAGGCAGTGAT
GCTAAACCTGAAGGGCAACATGGCCTATGGAGAAAGCATGGAGCTCAGAGCCTGGAGTA
[C.G]
GGGCACAGATAGGATTGAATAAATTGTGTAGAAAGACTTTGAAAACAATAAAGCAAAAGA
TGAATGAACGTTTTTTTAGACTTGAGGGACCAACAACCCCCAAACCCAGATTCTGCCA
GGTCCATGGGGAAGGAGAAGTTGCCTTGAGTGGAAGCCCCAAGTAGGGAGACTTACAGAA
AAGAAGTCAAGAGCACTGGCTCCAGGCAGAAATACTGATACCCTACTGGGGCTTCAGGC
TGAGCTCCTCCCTTCAAAATCACTTCATCTCTGAGCCTGTTTCTGCATCTGTGACAT

41303 CTCTGAGCCTGTTTCTGCATCTGTGACATAAGATGGTAAGATAAAGGTGGCTGTCTCACC
AATTATGTAAGGATTAAATGTGGAAGGACATAAAGTTGTATAGTGCTGCCATAGGGAC
AGTGTTCAGTAAACGTGACACATTCTTAGTATCACTAAGAATCAGGTTCTTGCCAGGCA
CCGTGGCTCATGCCTGTAATCCCAACACTCTGGGAGGCCTAGGTGGGAGGATGGCTTGA
CACAGGAGTTTGAGACCAGCCTGAGCAACATAGTGAGACACTGTCTTACAAAAA
[T.A]
AATAATAATAATTGTTTTTAATTAGATGGGCAGGGCACTGTGGCTCACACCTGTAATCCC
AGCACTTTGGGAGGCCAAGGCCGGAGGATTGCTTGAGGCCAGGAGTTCAGGAGCAGCCTG
GGCCACATTCTGTCTCTACAAAGAATAAAAAAGTTAACTGGGCATGGTGGCACATGCCT
GTAATCCAGCTACTCAAGAGGCTGAGGAGGAGGATTGCCTGAGCCAGGAGTTCAGAC
TGCAGTGAGCCTTGATCACACCACTGTACTACAGCTTGGGCAACAGAGTGAGACCTTGT

FIG.3-31

41305 CTGAGCCTGTTTCTGCATCTGTGACATAAGATGGTAAGATAAAGGTGGCTGTCTACCAA
TTATGTAAGGATTAAATGTGGAAAAGGACATAAAGTTGTATAGTGCTGCCATAGGGACAG
TGTTTCAGTAAACGTGACACATTCTTAGTATCACTAAGAATCAGGTTCTTGGCCAGGCACC
GTGGCTCATGCCTGTAATCCCAACTCTGGGAGGCCTAGGTCGGAGGATGGCTTGAACA
CAGGAGTTTGAGACCAGCCTGAGCAACATAGTGAGACACTGTCTCTACAAAAAAAATA
[-.A]
TAATAATAATTGTTTTTAATTAGATGGGCAGGGCACTGTGGCTCACACCTGTAATCCCAG
CACTTTGGGAGGCCAAGGCCGAGGATTGCTTGAGGCCAGGAGTTCAGGAGCAGCCTGGG
CCACATTCTGTCTCTACAAAGAATAAAAAAGTTAACTGGGCATGGTGGCACATGCCTGT
AATCCCAGCTACTCAAGAGGCTGAGGAGGAGGATTGCCTGAGCCCAGGAGTTCAAGACTG
CAGTGAGCCTTGATCACACCACTGTACTACAGCTTGGGCAACAGAGTGAGACCTTGTCTC

41457 CTAAGAATCAGGTTCTTGGCCAGGCACCGTGGCTCATGCCTGTAATCCCAACTCTGGG
AGGCCTAGGTCGGAGGATGGCTTGAACACAGGAGTTTGAGACCAGCCTGAGCAACATAGT
GAGACACTGTCTCTACAAAAAAAATAATAATAATTGTTTTTAATTAGATGGGCAG
GGCACTGTGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCCAAGGCCGAGGATTGCT
TGAGGCCAGGAGTTCAGGAGCAGCCTGGGCCACATTCTGTCTCTACAAAGAATAAAAA
[G.C]
TTAACTGGGCATGGTGGCACATGCCTGTAATCCCAGCTACTCAAGAGGCTGAGGAGGAGG
ATTGCCTGAGCCCAGGAGTTCAAGACTGCAGTGAGCCTTGATCACACCACTGTACTACAG
CTTGGGCAACAGAGTGAGACCTTGTCTCCAAAAAAAAGTTTGTTTTTTTTATCCACT
CTCCTCACCACAACTGAGTAAGTTAGAGCCCTCTCAGCTGGCATGTGTTGGAAACAG
TGCCCTCTCATTAAAGTGCTGCCCTCACTCCCATTCCTTGGCCTTGGTCAGTATGAT

43168 AGCTACTTGGGAGGCTGAGGCAGGAGAATCGCTTGAACCTGGAAGGCGGAGGTGCGAGTG
AGCCGAGATCGTGCCATTGCACTTCAGCCTGGGCGACAGAGCGAGACTCTGTCTCAAAAA
TAATAATAATAACAATAACTAGCCGGGCTGGTGGCACATGCCTGTAGTCCCAGTTACTC
AGGAGGCGGAGGCATGAGACTCAGGTGAAC TAGGGAGACAGAGGTTGCACTGAGCCAAGA
TCACACCACTGCACTCCAGCCTGGTTGACAGAGCGAGACTCTGTCTCAAAAAAAAATA
[A.-.T]
CCCATTGCTCATTTTTTTGGATACTAGTATAACTATCACTCTAAACCAGTTAGTACTTAA
ATCAAGCAGATATGGGAGATGGTGAATTACCATCTACAGTGTTGTATATATGTCACATA
CTGAGCATTATCAGCTAGTAGAATCTAGTTAATTGTTCTATGTGTGATGTATGCAGAGTT
CCCATTGTAATGTGTTTTTACTATGCTTAAATAAATGACTGATGTCAGCAACCCCAAAA
TGATACATCTGATGTAAGAGCCCTGTTCCCAATAATAACATCTAAACTATAGACATTG

43357 AGGCATGAGACTCAGGTGAACTAGGGAGACAGAGGTTGCAGTGAGCCAAGATCACACCAC
TGCACTCCAGCCTGGTTGACAGAGCGAGACTCTGTCTCAAAAAAAAATAATCCCATTTG
CTCATTTTTTGGATACTAGTATAACTATCACTCTAAACCAGTTAGTACTTAAATCAAGCA
GATATGGGAGATGGTGAATTACCATCTACAGTGTTGTATATATGTCACATACTGAGCAT
TATCAGCTAGTAGAATCTAGTTAATTGTTCTATGTGTGATGTATGCAGAGTTCCCATTTT
[T.G]
AATGTGTTTTTACTATGCTTAAATAAATGACTGATGTCAGCAACCCCAAAATGATACATC
TGATGTAAGAGCCCTGTTCCCAATAATAACATCTAAACTATAGACATTGGAATGAACA
GGTGCCCTAAGTTTCTCCCTCCAGGGTTTCTTGGCCGGTCTCTGAGGACTACACATCC
CTACTCCCGTCTTCTCATCTTCAGGCGCAGTAACAGTATCTCCAAGTCCCCTGGCCCC
AGCTCCCAAGGAGCCCTGCTGTTGAGCCGTGACATCAGCCGCTCAGAATCCCTTCGT

FIG.3-32

45664

CCAGCTTTCCTTGGCTTCCCCACCCCCAGGTGAAAGTGATGCGCAGCCTGGACCACCCC
AATGTGCTCAAGTTCATTGGTGTGCTGTACAAGGATAAGAAGCTGAACCTGCTGACAGAG
TACATTGAGGGGGGCACACTGAAGGACTTTCTGCGCAGTATGGTGAGCACACCACCCCAT
AGTCTCCAGGAGCCTTGGTGGTTGTGACACACCTATGCTATCACTACCCTAGGAGCTTA
AAGGGCAGAGGGGCCCTGCTTTCCTCCAAAGGACCATGCTGGGTGGGACTGAGCATACA
[T,C]
AGGGAGGCTTCACTGGGAGACCACATTGACCCATGGGGCCTGGACCACGAGTGGGACAGG
GCTCAACAGCCTCTGAAAATCATTCCCCATTCTGCAGGATCCGTTCCCTGGCAGCAGAA
GGTCAGGTTTGCCAAAGGAATCGCTCCGGAATGGTGAGTCCCAACAAACCTGCCAG
CAGGGCGAGAGTAGGGAGAGGTGTGAGAATTGTGGGCTTCACTGGAAGGTAGAGACCCCT
TCCTATGCAACTTGTGTGGGCTGGGTCAGCAGCTATTCAATTGAGTTTGTCTGTGCTCACTG

47549

AATTAGCTGGGCGTGGTGGTGCACGCCTGTAGTCCCAGCTACTCAGGAGGCCGAGGCAGG
AGAATAGCTTGAACCTGGGAGGCAGAAAGTTGCAGTGAGCCAAGATCACACCACTGCATT
CAGCCTGGGTGACAGAGTGAGACTTCATCTCAAAAAAAAAAAAAAGAGAGACTGATATG
GTTAGTACATTGGGGTGAATGCGGAGGGTCCAGGGAATGGAGCCCTGCATAGGGGGCTA
ATGAAACATTTAGATTTCTGAATTAAGGTAGTGGCTGTGGGGACAGGAGCCTGGGAGGC
[A,C]
GGGTGGAGTCAGAATGGAGAGACTGGTTGGCAATGAGGGAACAGGAGGAGGAGGAGGAGG
AGTTACGAGTGGCTTGAGGTGTCACTTACCAGACATTTGGGGGATGGGGGATAGCCGTGA
TTGTTGAGCAACTGGTTTGGGAAGAGCTAGCATTGATCCCTGCTGTTCTGTGCTAGCAGA
ACCTATCAGCATCTTCTGGGCAGGAACTGGCTCCATGAGACTGGCTTAGGGAGAGGCTG
CTAGTCACCTAATCTGCAGAGAAGGGGCAGCTGGAGCTGTGGGACAGAAGAGGCATCCAT

47908

GGAGTTACGAGTGGCTTGAGGTGTCACTTACCAGACATTTGGGGGATGGGGGATAGCCGT
GATTGTTGAGCAACTGGTTTGGGAAGAGCTAGCATTGATCCCTGCTGTTCTGTGCTAGCA
GAACCTATCAGCATCTTCTGGGCAGGAACTGGCTCCATGAGACTGGCTTAGGGAGAGGC
TGCTAGTCACCTAATCTGCAGAGAAGGGGCAGCTGGAGCTGTGGGACAGAAGAGGCATCC
ATGTAGCTGGTGGGGTGTCTCAGCTTGTGAAGAGGAGATGGCTTTGAGCAGGGCTGACA
[C,A]
TGAAAAGGCTGGAAGAAAAAACAGACACACAAGAGTCTCAGGATCAGGTAGCATAGGAA
AGTTGTGGACAGTCTTTGAGGAGCACTCCCTCAGGCAGGCAGGCAGGCAGGTGATGAGCT
ATAGCGATTGAGGAAGAGCTCCCTGGGTGTGTGAGCAGCTCCAGGAGCCTAAGGGATGAA
AGTAGTATTGCAGGGGGCTGGAGAGCAAGGAGTGGCTCCTTCTACATTTGCAAGGGAAGG
AGAAAGGAAGTTGCTCCTGAGAGTGGAAGAGTCAGTGGTGGAGGCCTGGAGAGGAGACA

52257

TTGTGAGGGGTAGAGGAGAGGAGAGACAAGGGATGGTTAGGATAATGAAGGAATGTTTTG
TTTTTGTGTTTTGTTTTGAGATGGAGTTTCACTCTGTCAACCAGGCTGGAGTGACAGAGGT
GCAATCTTGGCTCACTGCAGCCTCCGCCTCCAGGTTCAAGCAATCCTCCTGCCTCAGCC
TCCCAAGTAGCTGGGACTACAGGTGTGCGCCACCAGCCTGGCTAATTTTTGTATTTTCA
GTAGAGACAGGGTTTCGCCATATTGGCCAGGCTGGTCTCAAATGCCTGACCTCAGGTGAT
[C,A]
CACCCGCTTCAGCCTCCCAAAGTGTGAGATTACAGGCATGAGCTACCGTGCCTGGCCAT
GAAGGAAGATTTGTTTTAAAAAATTGTTTTCTTTAATTAATTGAACACCTCTGTTTCA
AGCACTGGGCTGGTGCCAGAGGGTTTCAGACATGAATCAGATCCAGCACCTCATAGAGCC
TTAATCTGGCACACACACAGCCACAAGGAGACACAGACAAGGCAGGGTAGGATGAGTG
GAAGCTAGGAGCAGATGCTGATTTGGAACACTTGGCTTCTGCAGTGAAGCCCTTCTTAG

FIG.3-33

- 54654 GGCCCCGGCCCCGGCCCCAGGCCAGGCAGTGGCGGCCAAGGACCACGCATCTACTTTCA
GAGCCCCCCCCGGGGCCGAGGAGAGGGCCCCGGCTGGCGGATGATGAGGGCCAGTGA
GGCGCCAAGGGAAGGTACCATCAAGTATGACCCAAGGAGCTACGGAAGCACCTCAACC
TAGAGGAGTGGATCCTGGAGCAGCTACGCGCCTCTACGACTGCCAGGAAGAGGAGATCT
CAGAACTAGAGATTGACGTGGATGAGCTCCTGGACATGGAGAGTGACGATGCCTGGGCTT
[T.C]
CAGGGTCAAGGAGCTGCTGGTTGACTGTTACAAACCCACAGAGGCCTTCATCTCTGGCCT
GCTGGACAAGATCCGGGCCATGCAGAAGCTGAGCACACCCAGAAGAAGTGAGGGTCCCC
GACCCAGGCGAACGGTGGCTCCCATAGGACAATCGCTACCCCCGACCTCGTAGCAACAG
CAATACCGGGGACCTGCGGCCAGGCCTGGTTCCATGAGCAGGGCTCCTCGTGCCCTG
GCCCAGGGGTCTCTTCCCCTGCCCCCTCAGTTTTCCACTTTTGGATTTTTTATTGTTAT
- 54679 GGCAGTGGCGGCCAAGGACCACGCATCTACTTTCAGAGCCCCCCCCGGGGCCGAGGAGA
GGGCCCGGGCTGGCGGATGATGAGGGCCAGTGAGGCGCCAAGGGAAGGTACCATCAA
GTATGACCCAAGGAGCTACGGAAGCACCTCAACCTAGAGGAGTGGATCCTGGAGCAGCT
CACGCGCCTCTACGACTGCCAGGAAGAGGAGATCTCAGAACTAGAGATTGACGTGGATGA
GCTCCTGGACATGGAGAGTGACGATGCCTGGGCTTCCAGGGTCAAGGAGCTGCTGGTTGA
[C.G]
TGTTACAAACCCACAGAGGCCTTCATCTCTGGCCTGCTGGACAAGATCCGGGCCATGCAG
AAGCTGAGCACACCCAGAAGAAGTGAGGGTCCCCGACCCAGCGAACGGTGGCTCCCAT
AGGACAATCGCTACCCCCGACCTCGTAGCAACAGCAATACCGGGGACCTGCGGCCAG
GCCTGGTTCCATGAGCAGGGCTCCTCGTGCCCTGGGCCAGGGGTCTCTTCCCCTGCCCC
CTCAGTTTTCCACTTTTGGATTTTTTATTGTTATTAACTGATGGGACTTTGTGTTTTT
- 54693 AGGACCACGCATCTACTTTCAGAGCCCCCCCCGGGGCCGAGGAGAGGGCCCGGGCTGGG
CGGATGATGAGGGCCAGTGAGGCGCCAAGGGAAGGTACCATCAAGTATGACCCAAGG
AGCTACGGAAGCACCTCAACCTAGAGGAGTGGATCCTGGAGCAGCTACGCGCCTCTACG
ACTGCCAGGAAGAGGAGATCTCAGAACTAGAGATTGACGTGGATGAGCTCCTGGACATGG
AGAGTGACGATGCCTGGGCTTCCAGGGTCAAGGAGCTGCTGGTTGACTGTTACAAACCCA
[A.C]
AGAGGCCTTCATCTCTGGCCTGCTGGACAAGATCCGGGCCATGCAGAAGCTGAGCACACC
CCAGAAGAAGTGAGGGTCCCCGACCCAGGCGAACGGTGGCTCCCATAGGACAATCGCTAC
CCCCGACCTCGTAGCAACAGCAATACCGGGGACCTGCGGCCAGGCCTGGTTCCATGA
GCAGGGCTCCTCGTGCCCTGGGCCAGGGGTCTCTTCCCCTGCCCCCTCAGTTTTCCACT
TTTGGATTTTTTATTGTTATTAACTGATGGGACTTTGTGTTTTTATATTGACTCTGCG
- 54706 TACTTTCAGAGCCCCCCCCGGGGCCGAGGAGAGGGCCCGGGCTGGCGGATGATGAGGG
CCAGTGAGGCGCCAAGGGAAGGTACCATCAAGTATGACCCAAGGAGCTACGGAAGCA
CCTCAACCTAGAGGAGTGGATCCTGGAGCAGCTACGCGCCTCTACGACTGCCAGGAAGA
GGAGATCTCAGAACTAGAGATTGACGTGGATGAGCTCCTGGACATGGAGAGTGACGATGC
CTGGGCTTCCAGGGTCAAGGAGCTGCTGGTTGACTGTTACAAACCCACAGAGGCCTTCAT
[T.C]
TCTGGCCTGCTGGACAAGATCCGGGCCATGCAGAAGCTGAGCACACCCAGAAGAAGTGA
GGGTCCCCGACCCAGGCGAACGGTGGCTCCCATAGGACAATCGCTACCCCCGACCTCGT
AGCAACAGCAATACCGGGGACCTGCGGCCAGGCCTGGTTCCATGAGCAGGGCTCCTCG
TGCCCTGGCCAGGGGTCTCTTCCCCTGCCCCCTCAGTTTTCCACTTTTGGATTTTTT
ATTGTTATTAACTGATGGGACTTTGTGTTTTTATATTGACTCTGCGGCACGGGCCCTT

FIG.3-34

- 54712 CAGAGCCCCCCCCGGGGCCGAGGAGAGGGCCCCGGGCTGGGCGGATGATGAGGGCCAGT
GAGGCGCCAAGGGAAGGTCACCATCAAGTATGACCCCAAGGAGCTACGGAAGCACCTCAA
CCTAGAGGAGTGGATCCTGGAGCAGCTCACGCGCCTCTACGACTGCCAGGAAGAGGAGAT
CTCAGAACTAGAGATTGACGTGGATGAGCTCCTGGACATGGAGAGTGACGATGCCTGGGC
TTCCAGGGTCAAGGAGCTGCTGGTTGACTGTTACAAACCCACAGAGGCCTTCATCTCTGG
[T.C]
CTGCTGGACAAGATCCGGGCCATGCAGAAGCTGAGCACACCCAGAGAAGTGAGGGTCC
CCGACCCAGGCGAACGGTGGCTCCCATAGGACAATCGCTACCCCCGACCTCGTAGCAAC
AGCAATACCGGGGACCCCTGCGGCCAGGCCTGGTTCCATGAGCAGGGCTCCTCGTGCCCC
TGGCCCAGGGGTCTCTTCCCTGCCCCCTCAGTTTTCCACTTTTGGATTTTTTATTGTT
ATTAACTGATGGGACTTTGTGTTTTATATTGACTCTGCGGCACGGGCCCTTTAATAAA
- 54799 GTATGACCCCAAGGAGCTACGGAAGCACCTCAACCTAGAGGAGTGGATCCTGGAGCAGCT
CACGCGCCTCTACGACTGCCAGGAAGAGGAGATCTCAGAACTAGAGATTGACGTGGATGA
GCTCCTGGACATGGAGAGTGACGATGCCTGGGCTTCCAGGGTCAAGGAGCTGCTGGTTGA
CTGTTACAAACCCACAGAGGCCTTCATCTCTGGCCTGCTGGACAAGATCCGGGCCATGCA
GAAGCTGAGCACACCCAGAGAAGTGAGGGTCCCCGACCCAGGCGAACGGTGGCTCCCA
[T.C]
AGGACAATCGCTACCCCCGACCTCGTAGCAACAGCAATACCGGGGGACCCCTGCGGCCAG
GCCTGGTTCCATGAGCAGGGCTCCTCGTGCCCTGGCCAGGGGTCTCTTCCCTGCCCC
CTCAGTTTTCCACTTTTGGATTTTTTATTGTTATTAAGCTGATGGGACTTTGTGTTTT
ATATTGACTCTGCGGCACGGGCCCTTTAATAAAGCGAGGTAGGGTACGCCTTTGGTGACG
CTCAAAAAAAAAAAAAAAAAATGATTTCAGCGGTCCACATTAGAGTTGAAATTTTCTGGT
- 54819 GGAAGCACCTCAACCTAGAGGAGTGGATCCTGGAGCAGCTCACGCGCCTCTACGACTGCC
AGGAAGAGGAGATCTCAGAACTAGAGATTGACGTGGATGAGCTCCTGGACATGGAGAGTG
ACGATGCCTGGGCTTCCAGGGTCAAGGAGCTGCTGGTTGACTGTTACAAACCCACAGAGG
CCTTCATCTCTGGCCTGCTGGACAAGATCCGGGCCATGCAGAAGCTGAGCACACCCAGA
AGAAGTGAGGGTCCCCGACCCAGGCGAACGGTGGCTCCCATAGGACAATCGCTACCCCC
[G.A]
ACCTCGTAGCAACAGCAATACCGGGGGACCCCTGCGGCCAGGCCTGGTTCCATGAGCAGGG
CTCCTCGTGCCCTGGCCAGGGGTCTCTTCCCTGCCCCCTCAGTTTTCCACTTTTGGG
TTTTTTTATTGTTATTAAGCTGATGGGACTTTGTGTTTTATATTGACTCTGCGGCACGG
GCCCTTTAATAAAGCGAGGTAGGGTACGCCTTTGGTGACGCTCAAAAAAAAAAAAAAAAA
TGATTTCCAGCGGTCCACATTAGAGTTGAAATTTTCTGGTGGGAGAACTATACCTTGT
- 55499 TTGTTTTCTAATACCTCTTGTCATTCTAAATATCTTTAATTTATTAATAATATATATAT
ACAGTATTGAATGCCTACTGTGTGCTAGGTACAGTTCTAAACACTTGGGTTACAGCAGCG
AACAAAATAAAGGTGCTTACCCTCATAGAACATAGATTCTAGCATGGTATCTACTGTATC
ATACAGTAGATACAATAAGTAACTATATTGAATATTAGAATGTGGCAGATGCTATGGAA
AAAGAGTCAAGACAAGTAAAGACGATTGTCAGGGTACCAGTTGCAATTTTAAATATGGT
[C.T]
GTCAGAGCAGGCCTCACTGAGGTGACATGACATTTAAGCATAAACATGGAGGAGGAGGAG
TAAGCCTGAGCTGTCTTAGGCTTCCGGGGCAGCCAAGCCATTTCCGTGGCACTAGGAGCC
TGGTGTTCGGATTCCACCTTTGATAACTGCATTTTCTAAGATATGGGAGGGAAGTTT
TTCTCCTATTGTTTTAAGTATTAAGTCCAGCTAGTCCAGCCTTGTATAGTGTTACCTA
ATCTTTATAGCAAATATATGAGGTACCGGTAAACATTATGCCCATTTCTCACAGAGGCACT

FIG.3-35

56825 ACTGATGGCTCAAAGGGTGTGAAAAAGTCAGTGATGCTCCCCCTTTCTACTCCAGATCCT
GTCCTTCCTGGAGCAAGGTTGAGGGAGTAGGTTTTGAAGAGTCCCTTAATATGTGGTGGA
ACAGGCCAGGAGTTAGAGAAAGGGCTGGCTTCTGTTTACCTGCTCACTGGCTCTAGCCAG
CCCAGGGACCACATCAATGTGAGAGGAAGCCTCCACCTCATGTTTTCAAACCTTAATACTG
GAGACTGGCTGAGAACTTACGGACAACATCCTTTCTGTCTGAAACAAACAGTCACAAGCA
[C,A]
AGGAAGAGGCTGGGGGACTAGAAAGAGGCCCTGCCCTCTAGAAAGCTCAGATCTTGGCTT
CTGTACTCATACTCGGGTGGGCTCCTTAGTCAGATGCCTAAAACATTTTGCCTAAAGCT
CGATGGGTCTGGAGGACAGTGTGGCTTGTCACAGGCCTAGAGTCTGAGGGAGGGGAGTG
GGAGTCTCAGCAATCTTTGGTCTTGGCTTCATGGCAACCACTGCTCACCTTCAACATG
CCTGGTTTAGGCAGCAGCTTGGGCTGGGAAGAGTGGTGGCAGAGTCTCAAAGCTGAGAT

58871 CGTCACCCACCACCAACCCCTGCCGCACTCCAGCCTTTAACAAGGGCTGTCTAGATATT
CATTTTAACTACCTCCACCTTGGAACAATTGCTGAAGGGGAGAGGATTTGCAATGACCA
ACCACCTTGTTGGGACGCCTGCACACCTGTCTTTCCTGCTTCAACCTGAAAGATTCTGA
TGATGATAATCTGGACACAGAAGCCGGGCACGGTGGCTCTAGCCTGTAATCTCAGCACTT
TGGGAGGCCTCAGCAGGTGGATCACCTGAGATCAAGAGTTTGAGAACAGCCTGACCAACA
[T,A]
GGTGAAACCCGCTCTCTACTAAAAATACAAAAATTAGCCAGGTGTGGTGGCACATACCTG
TAATCCCAGCTACTCTGGAGGCTGAGGCAGGAGAATCGCTTGAACCCACAAGGCAGAGGT
TGCAGTGAGGCGAGATCATGCCATTGCACTCCAGCCTGTGCAACAAGAGCCAACTCCAT
CTCAAAAAAAAAA

FIG.3-36

1

ISOLATED HUMAN KINASE PROTEINS, NUCLEIC ACID MOLECULES ENCODING HUMAN KINASE PROTEINS, AND USES THEREOF

FIELD OF THE INVENTION

The present invention is in the field of kinase proteins that are related to the serine/threonine kinase subfamily, recombinant DNA molecules, and protein production. The present invention specifically provides novel peptides and proteins that effect protein phosphorylation and nucleic acid molecules encoding such peptide and protein molecules, all of which are useful in the development of human therapeutics and diagnostic compositions and methods.

BACKGROUND OF THE INVENTION

Protein Kinases

Kinases regulate many different cell proliferation, differentiation, and signaling processes by adding phosphate groups to proteins. Uncontrolled signaling has been implicated in a variety of disease conditions including inflammation, cancer, arteriosclerosis, and psoriasis. Reversible protein phosphorylation is the main strategy for controlling activities of eukaryotic cells. It is estimated that more than 1000 of the 10,000 proteins active in a typical mammalian cell are phosphorylated. The high energy phosphate, which drives activation, is generally transferred from adenosine triphosphate molecules (ATP) to a particular protein by protein kinases and removed from that protein by protein phosphatases. Phosphorylation occurs in response to extracellular signals (hormones, neurotransmitters, growth and differentiation factors, etc), cell cycle checkpoints, and environmental or nutritional stresses and is roughly analogous to turning on a molecular switch. When the switch goes on, the appropriate protein kinase activates a metabolic enzyme, regulatory protein, receptor, cytoskeletal protein, ion channel or pump, or transcription factor.

The kinases comprise the largest known protein group, a superfamily of enzymes with widely varied functions and specificities. They are usually named after their substrate, their regulatory molecules, or some aspect of a mutant phenotype. With regard to substrates, the protein kinases may be roughly divided into two groups; those that phosphorylate tyrosine residues (protein tyrosine kinases, PTK) and those that phosphorylate serine or threonine residues (serine/threonine kinases, STK). A few protein kinases have dual specificity and phosphorylate threonine and tyrosine residues. Almost all kinases contain a similar 250-300 amino acid catalytic domain. The N-terminal domain, which contains subdomains I-IV, generally folds into a two-lobed structure, which binds and orients the ATP (or GTP) donor molecule. The larger C terminal lobe, which contains subdomains VI A-XI, binds the protein substrate and carries out the transfer of the gamma phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Subdomain V spans the two lobes.

The kinases may be categorized into families by the different amino acid sequences (generally between 5 and 100 residues) located on either side of, or inserted into loops of, the kinase domain. These added amino acid sequences allow the regulation of each kinase as it recognizes and interacts with its target protein. The primary structure of the kinase domains is conserved and can be further subdivided into 11 subdomains. Each of the 11 subdomains contains specific residues and motifs or patterns of amino acids that

2

are characteristic of that subdomain and are highly conserved (Hardie, G. and Hanks, S. (1995) *The Protein Kinase Facts Books*, Vol I:7-20 Academic Press, San Diego, Calif.).

The second messenger dependent protein kinases primarily mediate the effects of second messengers such as cyclic AMP (cAMP), cyclic GMP, inositol triphosphate, phosphatidylinositol, 3,4,5-triphosphate, cyclic-ADP-ribose, arachidonic acid, diacylglycerol and calcium-calmodulin. The cyclic-AMP dependent protein kinases (PKA) are important members of the STK family. Cyclic-AMP is an intracellular mediator of hormone action in all prokaryotic and animal cells that have been studied. Such hormone-induced cellular responses include thyroid hormone secretion, cortisol secretion, progesterone secretion, glycogen breakdown, bone resorption, and regulation of heart rate and force of heart muscle contraction. PKA is found in all animal cells and is thought to account for the effects of cyclic-AMP in most of these cells. Altered PKA expression is implicated in a variety of disorders and diseases including cancer, thyroid disorders, diabetes, atherosclerosis, and cardiovascular disease (Isselbacher, K. J. et al. (1994) *Harrison's Principles of Internal Medicine*, McGraw-Hill, New York, N.Y., pp. 416-431, 1887).

Calcium-calmodulin (CaM) dependent protein kinases are also members of STK family. Calmodulin is a calcium receptor that mediates many calcium regulated processes by binding to target proteins in response to the binding of calcium. The principle target protein in these processes is CaM dependent protein kinases. CaM-kinases are involved in regulation of smooth muscle contraction (MLC kinase), glycogen breakdown (phosphorylase kinase), and neurotransmission (CaM kinase I and CaM kinase II). CaM kinase I phosphorylates a variety of substrates including the neurotransmitter related proteins synapsin I and II, the gene transcription regulator, CREB, and the cystic fibrosis conductance regulator protein, CFTR (Haribabu, B. et al. (1995) *EMBO Journal* 14:3679-86). CaM II kinase also phosphorylates synapsin at different sites, and controls the synthesis of catecholamines in the brain through phosphorylation and activation of tyrosine hydroxylase. Many of the CaM kinases are activated by phosphorylation in addition to binding to CaM. The kinase may autophosphorylate itself, or be phosphorylated by another kinase as part of a "kinase cascade".

Another ligand-activated protein kinase is 5'-AMP-activated protein kinase (AMPK) (Gao, G. et al. (1996) *J. Biol. Chem.* 15:8675-81). Mammalian AMPK is a regulator of fatty acid and sterol synthesis through phosphorylation of the enzymes acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase and mediates responses of these pathways to cellular stresses such as heat shock and depletion of glucose and ATP. AMPK is a heterotrimeric complex comprised of a catalytic alpha subunit and two non-catalytic beta and gamma subunits that are believed to regulate the activity of the alpha subunit. Subunits of AMPK have a much wider distribution in non-lipogenic tissues such as brain, heart, spleen, and lung than expected. This distribution suggests that its role may extend beyond regulation of lipid metabolism alone.

The mitogen-activated protein kinases (MAP) are also members of the STK family. MAP kinases also regulate intracellular signaling pathways. They mediate signal transduction from the cell surface to the nucleus via phosphorylation cascades. Several subgroups have been identified, and each manifests different substrate specificities and responds to distinct extracellular stimuli (Egan, S. E. and Weinberg, R. A. (1993) *Nature* 365:781-783). MAP kinase signaling

pathways are present in mammalian cells as well as in yeast. The extracellular stimuli that activate mammalian pathways include epidermal growth factor (EGF), ultraviolet light, hyperosmolar medium, heat shock, endotoxic lipopolysaccharide (LPS), and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1).

PRK (proliferation-related kinase) is a serum/cytokine inducible STK that is involved in regulation of the cell cycle and cell proliferation in human megakaryotic cells (Li, B. et al. (1996) *J. Biol. Chem.* 271:19402-8). PRK is related to the polo (derived from humans polo gene) family of STKs implicated in cell division. PRK is downregulated in lung tumor tissue and may be a proto-oncogene whose deregulated expression in normal tissue leads to oncogenic transformation. Altered MAP kinase expression is implicated in a variety of disease conditions including cancer, inflammation, immune disorders, and disorders affecting growth and development.

The cyclin-dependent protein kinases (CDKs) are another group of STKs that control the progression of cells through the cell cycle. Cyclins are small regulatory proteins that act by binding to and activating CDKs that then trigger various phases of the cell cycle by phosphorylating and activating selected proteins involved in the mitotic process. CDKs are unique in that they require multiple inputs to become activated. In addition to the binding of cyclin, CDK activation requires the phosphorylation of a specific threonine residue and the dephosphorylation of a specific tyrosine residue.

Protein tyrosine kinases, PTKs, specifically phosphorylate tyrosine residues on their target proteins and may be divided into transmembrane, receptor PTKs and nontransmembrane, non-receptor PTKs. Transmembrane protein-tyrosine kinases are receptors for most growth factors. Binding of growth factor to the receptor activates the transfer of a phosphate group from ATP to selected tyrosine side chains of the receptor and other specific proteins. Growth factors (GF) associated with receptor PTKs include; epidermal GF, platelet-derived GF, fibroblast GF, hepatocyte GF, insulin and insulin-like GFs, nerve GF, vascular endothelial GF, and macrophage colony stimulating factor.

Non-receptor PTKs lack transmembrane regions and, instead, form complexes with the intracellular regions of cell surface receptors. Such receptors that function through non-receptor PTKs include those for cytokines, hormones (growth hormone and prolactin) and antigen-specific receptors on T and B lymphocytes.

Many of these PTKs were first identified as the products of mutant oncogenes in cancer cells where their activation was no longer subject to normal cellular controls. In fact, about one third of the known oncogenes encode PTKs, and it is well known that cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (Carbonneau H and Tonks NK (1992) *Annu. Rev. Cell. Biol.* 8:463-93). Regulation of PTK activity may therefore be an important strategy in controlling some types of cancer.

LIM Domain Kinases

The novel human protein, and encoding gene, provided by the present invention is related to the family of serine/threonine kinases in general, particularly LIM domain kinases (LIMK), and shows the highest degree of similarity to LIMK2, and the LIMK2b isoform (Genbank gi8051618) in particular (see the amino acid sequence alignment of the protein of the present invention against LIMK2b provided in

FIG. 2). LIMK proteins generally have serine/threonine kinase activity. The protein of the present invention may be a novel alternative splice form of the art-known protein provided in Genbank gi805161; however, the structure of the gene provided by the present invention is different from the art-known gene of gi8051618 and the first exon of the gene of the present invention is novel, suggesting a novel gene rather than an alternative splice form. Furthermore, the protein of the present invention lacks an LIM domain relative to gi8051618. The protein of the present invention does contain the kinase catalytic domain.

Approximately 40 LIM proteins, named for the LIM domains they contain, are known to exist in eukaryotes. LIM domains are conserved, cysteine-rich structures that contain 2 zinc fingers that are thought to modulate protein-protein interactions. LIMK1 and LIMK2 are members of a LIM subfamily characterized by 2 N-terminal LIM domains and a C-terminal protein kinase domain. LIMK1 and LIMK2 mRNA expression varies greatly between different tissues. The protein kinase domains of LIMK1 and LIMK2 contain a unique sequence motif comprising Asp-Leu-Asn-Ser-His-Asn in subdomain VIB and a strongly basic insert between subdomains VII and VIII (Okano et al., *J. Biol. Chem.* 270 (52), 31321-31330 (1995)). The protein kinase domain present in LIMKs is significantly different than other kinase domains, sharing about 32% identity.

LIMK is activated by ROCK (a downstream effector of Rho) via phosphorylation. LIMK then phosphorylates cofilin, which inhibits its actin-depolymerizing activity, thereby leading to Rho-induced reorganization of the actin cytoskeleton (Maekawa et al., *Science* 285: 895-898, 1999).

The LIMK2a and LIMK2b alternative transcript forms are differentially expressed in a tissue-specific manner and are generated by variation in transcriptional initiation utilizing alternative promoters. LIMK2a contains 2 LIM domains, a PDZ domain (a domain that functions in protein-protein interactions targeting the protein to the submembranous compartment), and a kinase domain; whereas LIMK2b just has 1.5 LIM domains. Alteration of LIMK2a and LIMK2b regulation has been observed in some cancer cell lines (Osada et al., *Biochem. Biophys. Res. Commun.* 229: 582-589, 1996).

For a further review of LIMK proteins, see Nomoto et al., *Gene* 236 (2), 259-271 (1999).

Kinase proteins, particularly members of the serine/threonine kinase subfamily, are a major target for drug action and development. Accordingly, it is valuable to the field of pharmaceutical development to identify and characterize previously unknown members of this subfamily of kinase proteins. The present invention advances the state of the art by providing previously unidentified human kinase proteins that have homology to members of the serine/threonine kinase subfamily.

SUMMARY OF THE INVENTION

The present invention is based in part on the identification of amino acid sequences of human kinase peptides and proteins that are related to the serine/threonine kinase subfamily, as well as allelic variants and other mammalian orthologs thereof. These unique peptide sequences, and nucleic acid sequences that encode these peptides, can be used as models for the development of human therapeutic targets, aid in the identification of therapeutic proteins, and serve as targets for the development of human therapeutic agents that modulate kinase activity in cells and tissues that express the kinase. Experimental data as provided in FIG. 1

5

indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland.

DESCRIPTION OF THE FIGURE SHEETS

FIG. 1 provides the nucleotide sequence of a cDNA molecule that encodes the kinase protein of the present invention. (SEQ ID NO:1) In addition, structure and functional information is provided, such as ATG start, stop and tissue distribution, where available, that allows one to readily determine specific uses of inventions based on this molecular sequence. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland.

FIG. 2 provides the predicted amino acid sequence of the kinase of the present invention. (SEQ ID NO:2) In addition structure and functional information such as protein family, function, and modification sites is provided where available, allowing one to readily determine specific uses of inventions based on this molecular sequence.

FIG. 3 provides genomic sequences that span the gene encoding the kinase protein of the present invention. (SEQ ID NO:3) In addition structure and functional information, such as intron/exon structure, promoter location, etc., is provided where available, allowing one to readily determine specific uses of inventions based on this molecular sequence. As illustrated in FIG. 3, SNPs were identified at 42 different nucleotide positions.

DETAILED DESCRIPTION OF THE INVENTION

General Description

The present invention is based on the sequencing of the human genome. During the sequencing and assembly of the human genome, analysis of the sequence information revealed previously unidentified fragments of the human genome that encode peptides that share structural and/or sequence homology to protein/peptide/domains identified and characterized within the art as being a kinase protein or part of a kinase protein and are related to the serine/threonine kinase subfamily. Utilizing these sequences, additional genomic sequences were assembled and transcript and/or cDNA sequences were isolated and characterized. Based on this analysis, the present invention provides amino acid sequences of human kinase peptides and proteins that are related to the serine/threonine kinase subfamily, nucleic acid sequences in the form of transcript sequences, cDNA sequences and/or genomic sequences that encode these kinase peptides and proteins, nucleic acid variation (allelic information), tissue distribution of expression, and information about the closest art known protein/peptide/domain that has structural or sequence homology to the kinase of the present invention.

In addition to being previously unknown, the peptides that are provided in the present invention are selected based on their ability to be used for the development of commercially important products and services. Specifically, the present peptides are selected based on homology and/or structural relatedness to known kinase proteins of the serine/threonine kinase subfamily and the expression pattern observed. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. The art has clearly established the commercial importance of

6

members of this family of proteins and proteins that have expression patterns similar to that of the present gene. Some of the more specific features of the peptides of the present invention, and the uses thereof, are described herein, particularly in the Background of the Invention and in the annotation provided in the Figures, and/or are known within the art for each of the known serine/threonine kinase family or subfamily of kinase proteins.

Specific Embodiments

Peptide Molecules

The present invention provides nucleic acid sequences that encode protein molecules that have been identified as being members of the kinase family of proteins and are related to the serine/threonine kinase subfamily (protein sequences are provided in FIG. 2, transcript/cDNA sequences are provided in FIG. 1 and genomic sequences are provided in FIG. 3). The peptide sequences provided in FIG. 2, as well as the obvious variants described herein, particularly allelic variants as identified herein and using the information in FIG. 3, will be referred herein as the kinase peptides of the present invention, kinase peptides, or peptides/proteins of the present invention.

The present invention provides isolated peptide and protein molecules that consist of, consist essentially of, or comprise the amino acid sequences of the kinase peptides disclosed in the FIG. 2, (encoded by the nucleic acid molecule shown in FIG. 1, transcript/cDNA or FIG. 3, genomic sequence), as well as all obvious variants of these peptides that are within the art to make and use. Some of these variants are described in detail below.

As used herein, a peptide is said to be "isolated" or "purified" when it is substantially free of cellular material or free of chemical precursors or other chemicals. The peptides of the present invention can be purified to homogeneity or other degrees of purity. The level of purification will be based on the intended use. The critical feature is that the preparation allows for the desired function of the peptide, even if in the presence of considerable amounts of other components (the features of an isolated nucleic acid molecule is discussed below).

In some uses, "substantially free of cellular material" includes preparations of the peptide having less than about 30% (by dry weight) other proteins (i.e., contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When the peptide is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of the peptide in which it is separated from chemical precursors or other chemicals that are involved in its synthesis. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of the kinase peptide having less than about 30% (by dry weight) chemical precursors or other chemicals, less than about 20% chemical precursors or other chemicals, less than about 10% chemical precursors or other chemicals, or less than about 5% chemical precursors or other chemicals.

The isolated kinase peptide can be purified from cells that naturally express it, purified from cells that have been altered to express it (recombinant), or synthesized using known protein synthesis methods. Experimental data as

provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. For example, a nucleic acid molecule encoding the kinase peptide is cloned into an expression vector, the expression vector introduced into a host cell and the protein expressed in the host cell. The protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Many of these techniques are described in detail below.

Accordingly, the present invention provides proteins that consist of the amino acid sequences provided in FIG. 2 (SEQ ID NO:2), for example, proteins encoded by the transcript/cDNA nucleic acid sequences shown in FIG. 1 (SEQ ID NO:1) and the genomic sequences provided in FIG. 3 (SEQ ID NO:3). The amino acid sequence of such a protein is provided in FIG. 2. A protein consists of an amino acid sequence when the amino acid sequence is the final amino acid sequence of the protein.

The present invention further provides proteins that consist essentially of the amino acid sequences provided in FIG. 2 (SEQ ID NO:2), for example, proteins encoded by the transcript/cDNA nucleic acid sequences shown in FIG. 1 (SEQ ID NO:1) and the genomic sequences provided in FIG. 3 (SEQ ID NO:3). A protein consists essentially of an amino acid sequence when such an amino acid sequence is present with only a few additional amino acid residues, for example from about 1 to about 100 or so additional residues, typically from 1 to about 20 additional residues in the final protein.

The present invention further provides proteins that comprise the amino acid sequences provided in FIG. 2 (SEQ ID NO:2), for example, proteins encoded by the transcript/cDNA nucleic acid sequences shown in FIG. 1 (SEQ ID NO:1) and the genomic sequences provided in FIG. 3 (SEQ ID NO:3). A protein comprises an amino acid sequence when the amino acid sequence is at least part of the final amino acid sequence of the protein. In such a fashion, the protein can be only the peptide or have additional amino acid molecules, such as amino acid residues (contiguous encoded sequence) that are naturally associated with it or heterologous amino acid residues/peptide sequences. Such a protein can have a few additional amino acid residues or can comprise several hundred or more additional amino acids. The preferred classes of proteins that are comprised of the kinase peptides of the present invention are the naturally occurring mature proteins. A brief description of how various types of these proteins can be made/isolated is provided below.

The kinase peptides of the present invention can be attached to heterologous sequences to form chimeric or fusion proteins. Such chimeric and fusion proteins comprise a kinase peptide operatively linked to a heterologous protein having an amino acid sequence not substantially homologous to the kinase peptide. "Operatively linked" indicates that the kinase peptide and the heterologous protein are fused in-frame. The heterologous protein can be fused to the N-terminus or C-terminus of the kinase peptide.

In some uses, the fusion protein does not affect the activity of the kinase peptide per se. For example, the fusion protein can include, but is not limited to, enzymatic fusion proteins, for example beta-galactosidase fusions, yeast two-hybrid GAL fusions, poly-His fusions, MYC-tagged, HI-tagged and Ig fusions. Such fusion proteins, particularly poly-His fusions, can facilitate the purification of recombinant kinase peptide. In certain host cells (e.g., mammalian host cells), expression and/or secretion of a protein can be increased by using a heterologous signal sequence.

A chimeric or fusion protein can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different protein sequences are ligated together in-frame in accordance with conventional techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see Ausubel et al., *Current Protocols in Molecular Biology*, 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST protein). A kinase peptide-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the kinase peptide.

As mentioned above, the present invention also provides and enables obvious variants of the amino acid sequence of the proteins of the present invention, such as naturally occurring mature forms of the peptide, allelic/sequence variants of the peptides, non-naturally occurring recombinantly derived variants of the peptides, and orthologs and paralogs of the peptides. Such variants can readily be generated using art-known techniques in the fields of recombinant nucleic acid technology and protein biochemistry. It is understood, however, that variants exclude any amino acid sequences disclosed prior to the invention.

Such variants can readily be identified/made using molecular techniques and the sequence information disclosed herein. Further, such variants can readily be distinguished from other peptides based on sequence and/or structural homology to the kinase peptides of the present invention. The degree of homology/identity present will be based primarily on whether the peptide is a functional variant or non-functional variant, the amount of divergence present in the paralog family and the evolutionary distance between the orthologs.

To determine the percent identity of two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% or more of the length of a reference sequence is aligned for comparison purposes. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm. (*Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part 1, Griffin, A. M., and Griffin, H. G.,

eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (Devereux, J., et al., *Nucleic Acids Res.* 12(1):387 (1984)) (available at <http://www.gcg.com>), using a NWS-gapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Myers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against sequence databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (*J. Mol. Biol.* 215:403-10 (1990)). BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (*Nucleic Acids Res.* 25(17):3389-3402 (1997)). When utilizing BLAST and gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Full-length pre-processed forms, as well as mature processed forms, of proteins that comprise one of the peptides of the present invention can readily be identified as having complete sequence identity to one of the kinase peptides of the present invention as well as being encoded by the same genetic locus as the kinase peptide provided herein. The gene encoding the novel kinase protein of the present invention is located on a genome component that has been mapped to human chromosome 22 (as indicated in FIG. 3), which is supported by multiple lines of evidence, such as STS and BAC map data.

Allelic variants of a kinase peptide can readily be identified as being a human protein having a high degree (significant) of sequence homology/identity to at least a portion of the kinase peptide as well as being encoded by the same genetic locus as the kinase peptide provided herein. Genetic locus can readily be determined based on the genomic information provided in FIG. 3, such as the genomic sequence mapped to the reference human. The gene encoding the novel kinase protein of the present invention is located on a genome component that has been mapped to human chromosome 22 (as indicated in FIG. 3), which is supported by multiple lines of evidence, such as STS and BAC map data. As used herein, two proteins (or a region of

the proteins) have significant homology when the amino acid sequences are typically at least about 70-80%, 80-90%, and more typically at least about 90-95% or more homologous. A significantly homologous amino acid sequence, according to the present invention, will be encoded by a nucleic acid sequence that will hybridize to a kinase peptide encoding nucleic acid molecule under stringent conditions as more fully described below.

FIG. 3 provides information on SNPs that have been found in the gene encoding the kinase protein of the present invention. SNPs were identified at 42 different nucleotide positions. Some of these SNPs, which are located outside the ORF and in introns, may affect gene transcription.

Paralogs of a kinase peptide can readily be identified as having some degree of significant sequence homology/identity to at least a portion of the kinase peptide, as being encoded by a gene from humans, and as having similar activity or function. Two proteins will typically be considered paralogs when the amino acid sequences are typically at least about 60% or greater, and more typically at least about 70% or greater homology through a given region or domain. Such paralogs will be encoded by a nucleic acid sequence that will hybridize to a kinase peptide encoding nucleic acid molecule under moderate to stringent conditions as more fully described below.

Orthologs of a kinase peptide can readily be identified as having some degree of significant sequence homology/identity to at least a portion of the kinase peptide as well as being encoded by a gene from another organism. Preferred orthologs will be isolated from mammals, preferably primates, for the development of human therapeutic targets and agents. Such orthologs will be encoded by a nucleic acid sequence that will hybridize to a kinase peptide encoding nucleic acid molecule under moderate to stringent conditions, as more fully described below, depending on the degree of relatedness of the two organisms yielding the proteins.

Non-naturally occurring variants of the kinase peptides of the present invention can readily be generated using recombinant techniques. Such variants include, but are not limited to deletions, additions and substitutions in the amino acid sequence of the kinase peptide. For example, one class of substitutions are conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a kinase peptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al., *Science* 247:1306-1310 (1990).

Variant kinase peptides can be fully functional or can lack function in one or more activities, e.g. ability to bind substrate, ability to phosphorylate substrate, ability to mediate signaling, etc. Fully functional variants typically contain only conservative variation or variation in non-critical residues or in non-critical regions. FIG. 2 provides the result of protein analysis and can be used to identify critical domains/regions. Functional variants can also contain substitution of similar amino acids that result in no change or an insignificant change in function. Alternatively, such substitutions may positively or negatively affect function to some degree.

Non-functional variants typically contain one or more non-conservative amino acid substitutions, deletions, insertions, inversions, or truncation or a substitution, insertion, inversion, or deletion in a critical residue or critical region:

Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham et al., *Science* 244:1081-1085 (1989)), particularly using the results provided in FIG. 2. The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as kinase activity or in assays such as an in vitro proliferative activity. Sites that are critical for binding partner/substrate binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith et al., *J. Mol. Biol.* 224:899-904 (1992); de Vos et al. *Science* 255:306-312 (1992)).

The present invention further provides fragments of the kinase peptides, in addition to proteins and peptides that comprise and consist of such fragments, particularly those comprising the residues identified in FIG. 2. The fragments to which the invention pertains, however, are not to be construed as encompassing fragments that may be disclosed publicly prior to the present invention.

As used herein, a fragment comprises at least 8, 10, 12, 14, 16, or more contiguous amino acid residues from a kinase peptide. Such fragments can be chosen based on the ability to retain one or more of the biological activities of the kinase peptide or could be chosen for the ability to perform a function, e.g. bind a substrate or act as an immunogen. Particularly important fragments are biologically active fragments, peptides that are, for example, about 8 or more amino acids in length. Such fragments will typically comprise a domain or motif of the kinase peptide, e.g., active site, a transmembrane domain or a substrate-binding domain. Further, possible fragments include, but are not limited to, domain or motif containing fragments, soluble peptide fragments, and fragments containing immunogenic structures. Predicted domains and functional sites are readily identifiable by computer programs well known and readily available to those of skill in the art (e.g., PROSITE analysis). The results of one such analysis are provided in FIG. 2.

Polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art. Common modifications that occur naturally in kinase peptides are described in basic texts, detailed monographs, and the research literature, and they are well known to those of skill in the art (some of these features are identified in FIG. 2).

Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pro-

teolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as *Proteins—Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York 1-12 (1983); Seifter et al. (*Meth. Enzymol.* 182: 626-646 (1990)) and Rattan et al. (*Ann. N.Y. Acad. Sci.* 663:48-62 (1992)).

Accordingly, the kinase peptides of the present invention also encompass derivatives or analogs in which a substituted amino acid residue is not one encoded by the genetic code, in which a substituent group is included, in which the mature kinase peptide is fused with another compound, such as a compound to increase the half-life of the kinase peptide (for example, polyethylene glycol), or in which the additional amino acids are fused to the mature kinase peptide, such as a leader or secretory sequence or a sequence for purification of the mature kinase peptide or a pro-protein sequence.

Protein/Peptide Uses

The proteins of the present invention can be used in substantial and specific assays related to the functional information provided in the Figures; to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its binding partner or ligand) in biological fluids; and as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state). Where the protein binds or potentially binds to another protein or ligand (such as, for example, in a kinase-effector protein interaction or kinase-ligand interaction), the protein can be used to identify the binding partner/ligand so as to develop a system to identify inhibitors of the binding interaction. Any or all of these uses are capable of being developed into reagent grade or kit format for commercialization as commercial products.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

The potential uses of the peptides of the present invention are based primarily on the source of the protein as well as the class/action of the protein. For example, kinases isolated from humans and their human/mammalian orthologs serve as targets for identifying agents for use in mammalian therapeutic applications, e.g. a human drug, particularly in modulating a biological or pathological response in a cell or tissue that expresses the kinase. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant

brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain. A large percentage of pharmaceutical agents are being developed that modulate the activity of kinase proteins, particularly members of the serine/threonine kinase subfamily (see Background of the Invention). The structural and functional information provided in the Background and Figures provide specific and substantial uses for the molecules of the present invention, particularly in combination with the expression information provided in FIG. 1. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. Such uses can readily be determined using the information provided herein, that which is known in the art, and routine experimentation.

The proteins of the present invention (including variants and fragments that may have been disclosed prior to the present invention) are useful for biological assays related to kinases that are related to members of the serine/threonine kinase subfamily. Such assays involve any of the known kinase functions or activities or properties useful for diagnosis and treatment of kinase-related conditions that are specific for the subfamily of kinases that the one of the present invention belongs to, particularly in cells and tissues that express the kinase. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain.

The proteins of the present invention are also useful in drug screening assays, in cell-based or cell-free systems. Cell-based systems can be native, i.e., cells that normally express the kinase, as a biopsy or expanded in cell culture. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. In an alternate embodiment, cell-based assays involve recombinant host cells expressing the kinase protein.

The polypeptides can be used to identify compounds that modulate kinase activity of the protein in its natural state or an altered form that causes a specific disease or pathology associated with the kinase. Both the kinases of the present invention and appropriate variants and fragments can be used in high-throughput screens to assay candidate compounds for the ability to bind to the kinase. These compounds can be further screened against a functional kinase to determine the effect of the compound on the kinase activity. Further, these compounds can be tested in animal or invertebrate systems to determine activity/effectiveness. Compounds can be identified that activate (agonist) or inactivate (antagonist) the kinase to a desired degree.

Further, the proteins of the present invention can be used to screen a compound for the ability to stimulate or inhibit interaction between the kinase protein and a molecule that normally interacts with the kinase protein, e.g. a substrate or a component of the signal pathway that the kinase protein normally interacts (for example, another kinase). Such assays typically include the steps of combining the kinase protein with a candidate compound under conditions that allow the kinase protein, or fragment, to interact with the target molecule, and to detect the formation of a complex between the protein and the target or to detect the biochemical consequence of the interaction with the kinase protein and the target, such as any of the associated effects of signal

transduction such as protein phosphorylation, cAMP turnover, and adenylate cyclase activation, etc.

Candidate compounds include, for example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, e.g., Lam et al., *Nature* 354:82-84 (1991); Houghten et al., *Nature* 354:84-86 (1991)) and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids; 2) phosphopeptides (e.g., members of random and partially degenerate, directed phosphopeptide libraries, see, e.g., Songyang et al., *Cell* 72:767-778 (1993)); 3) antibodies (e.g., polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, F(ab')₂, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (e.g., molecules obtained from combinatorial and natural product libraries).

One candidate compound is a soluble fragment of the receptor that competes for substrate binding. Other candidate compounds include mutant kinases or appropriate fragments containing mutations that affect kinase function and thus compete for substrate. Accordingly, a fragment that competes for substrate, for example with a higher affinity, or a fragment that binds substrate but does not allow release, is encompassed by the invention.

The invention further includes other end point assays to identify compounds that modulate (stimulate or inhibit) kinase activity. The assays typically involve an assay of events in the signal transduction pathway that indicate kinase activity. Thus, the phosphorylation of a substrate, activation of a protein, a change in the expression of genes that are up- or down-regulated in response to the kinase protein dependent signal cascade can be assayed.

Any of the biological or biochemical functions mediated by the kinase can be used as an endpoint assay. These include all of the biochemical or biochemical/biological events described herein, in the references cited herein, incorporated by reference for these endpoint assay targets, and other functions known to those of ordinary skill in the art or that can be readily identified using the information provided in the Figures, particularly FIG. 2. Specifically, a biological function of a cell or tissues that expresses the kinase can be assayed. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain.

Binding and/or activating compounds can also be screened by using chimeric kinase proteins in which the amino terminal extracellular domain, or parts thereof, the entire transmembrane domain or subregions, such as any of the seven transmembrane segments or any of the intracellular or extracellular loops and the carboxy terminal intracellular domain, or parts thereof, can be replaced by heterologous domains or subregions. For example, a substrate-binding region can be used that interacts with a different substrate than that which is recognized by the native kinase. Accordingly, a different set of signal transduction components is available as an end-point assay for activation. This allows for assays to be performed in other than the specific host cell from which the kinase is derived.

The proteins of the present invention are also useful in competition binding assays in methods designed to discover compounds that interact with the kinase (e.g. binding part-

ners and/or ligands). Thus, a compound is exposed to a kinase polypeptide under conditions that allow the compound to bind or to otherwise interact with the polypeptide. Soluble kinase polypeptide is also added to the mixture. If the test compound interacts with the soluble kinase polypeptide, it decreases the amount of complex formed or activity from the kinase target. This type of assay is particularly useful in cases in which compounds are sought that interact with specific regions of the kinase. Thus, the soluble polypeptide that competes with the target kinase region is designed to contain peptide sequences corresponding to the region of interest.

To perform cell free drug screening assays, it is sometimes desirable to immobilize either the kinase protein, or fragment, or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay.

Techniques for immobilizing proteins on matrices can be used in the drug screening assays. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (e.g., ³⁵S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of kinase-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques. For example, either the polypeptide or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin using techniques well known in the art. Alternatively, antibodies reactive with the protein but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and the protein trapped in the wells by antibody conjugation. Preparations of a kinase-binding protein and a candidate compound are incubated in the kinase protein-presenting wells and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the kinase protein target molecule, or which are reactive with kinase protein and compete with the target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

Agents that modulate one of the kinases of the present invention can be identified using one or more of the above assays, alone or in combination. It is generally preferable to use a cell-based or cell free system first and then confirm activity in an animal or other model system. Such model systems are well known in the art and can readily be employed in this context.

Modulators of kinase protein activity identified according to these drug screening assays can be used to treat a subject with a disorder mediated by the kinase pathway, by treating cells or tissues that express the kinase. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. These methods of treatment include the steps of administering a modulator of

kinase activity in a pharmaceutical composition to a subject in need of such treatment, the modulator being identified as described herein.

In yet another aspect of the invention, the kinase proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Pat. No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693/1696; and Brent WO94110300), to identify other proteins, which bind to or interact with the kinase and are involved in kinase activity. Such kinase-binding proteins are also likely to be involved in the propagation of signals by the kinase proteins or kinase targets as, for example, downstream elements of a kinase-mediated signaling pathway. Alternatively, such kinase-binding proteins are likely to be kinase inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a kinase protein is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming a kinase-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the kinase protein.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., a kinase-modulating agent, an antisense kinase nucleic acid molecule, a kinase-specific antibody, or a kinase-binding partner) can be used in an animal or other model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal or other model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

The kinase proteins of the present invention are also useful to provide a target for diagnosing a disease or predisposition to disease mediated by the peptide. Accordingly, the invention provides methods for detecting the presence, or levels of, the protein (or encoding mRNA) in a cell, tissue, or organism. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. The method involves contacting a biological sample with a compound capable of interacting with the kinase protein such that the interaction can be detected. Such an assay can be provided in a single detection format or a multi-detection format such as an antibody chip array.

One agent for detecting a protein in a sample is an antibody capable of selectively binding to protein. A bio-

logical sample includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject.

The peptides of the present invention also provide targets for diagnosing active protein activity, disease, or predisposition to disease, in a patient having a variant peptide, particularly activities and conditions that are known for other members of the family of proteins to which the present one belongs. Thus, the peptide can be isolated from a biological sample and assayed for the presence of a genetic mutation that results in aberrant peptide. This includes amino acid substitution, deletion, insertion, rearrangement, (as the result of aberrant splicing events), and inappropriate post-translational modification. Analytic methods include altered electrophoretic mobility, altered tryptic peptide digest, altered kinase activity in cell-based or cell-free assay, alteration in substrate or antibody-binding pattern, altered isoelectric point, direct amino acid sequencing, and any other of the known assay techniques useful for detecting mutations in a protein. Such an assay can be provided in a single detection format or a multi-detection format such as an antibody chip array.

In vitro techniques for detection of peptide include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence using a detection reagent, such as an antibody or protein binding agent. Alternatively, the peptide can be detected in vivo in a subject by introducing into the subject a labeled anti-peptide antibody or other types of detection agent. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. Particularly useful are methods that detect the allelic variant of a peptide expressed in a subject and methods which detect fragments of a peptide in a sample.

The peptides are also useful in pharmacogenomic analysis. Pharmacogenomics deal with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Eichelbaum, M. (*Clin. Exp. Pharmacol. Physiol.* 23(10-11):983-985 (1996)), and Linder, M. W. (*Clin. Chem.* 43(2):254-266 (1997)). The clinical outcomes of these variations result in severe toxicity of therapeutic drugs in certain individuals or therapeutic failure of drugs in certain individuals as a result of individual variation in metabolism. Thus, the genotype of the individual can determine the way a therapeutic compound acts on the body or the way the body metabolizes the compound. Further, the activity of drug metabolizing enzymes effects both the intensity and duration of drug action. Thus, the pharmacogenomics of the individual permit the selection of effective compounds and effective dosages of such compounds for prophylactic or therapeutic treatment based on the individual's genotype. The discovery of genetic polymorphisms in some drug metabolizing enzymes has explained why some patients do not obtain the expected drug effects, show an exaggerated drug effect, or experience serious toxicity from standard drug dosages. Polymorphisms can be expressed in the phenotype of the extensive metabolizer and the phenotype of the poor metabolizer. Accordingly, genetic polymorphism may lead to allelic protein variants of the kinase protein in which one or more of the kinase functions in one population is different from those in another population. The peptides thus allow a target to ascertain a genetic predisposition that can affect treatment modality. Thus, in a ligand-based treatment, polymorphism may give rise to amino terminal extracellular domains and/or other substrate-binding regions that are

more or less active in substrate binding, and kinase activation. Accordingly, substrate dosage would necessarily be modified to maximize the therapeutic effect within a given population containing a polymorphism. As an alternative to genotyping, specific polymorphic peptides could be identified.

The peptides are also useful for treating a disorder characterized by an absence of, inappropriate, or unwanted expression of the protein. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. Accordingly, methods for treatment include the use of the kinase protein or fragments.

Antibodies

The invention also provides antibodies that selectively bind to one of the peptides of the present invention, a protein comprising such a peptide, as well as variants and fragments thereof. As used herein, an antibody selectively binds a target peptide when it binds the target peptide and does not significantly bind to unrelated proteins. An antibody is still considered to selectively bind a peptide even if it also binds to other proteins that are not substantially homologous with the target peptide so long as such proteins share homology with a fragment or domain of the peptide target of the antibody. In this case, it would be understood that antibody binding to the peptide is still selective despite some degree of cross-reactivity.

As used herein, an antibody is defined in terms consistent with that recognized within the art: they are multi-subunit proteins produced by a mammalian organism in response to an antigen challenge. The antibodies of the present invention include polyclonal antibodies and monoclonal antibodies, as well as fragments of such antibodies, including, but not limited to, Fab or F(ab)₂, and Fv fragments.

Many methods are known for generating and/or identifying antibodies to a given target peptide. Several such methods are described by Harlow, Antibodies, Cold Spring Harbor Press, (1989).

In general, to generate antibodies, an isolated peptide is used as an immunogen and is administered to a mammalian organism, such as a rat, rabbit or mouse. The full-length protein, an antigenic peptide fragment or a fusion protein can be used. Particularly important fragments are those covering functional domains, such as the domains identified in FIG. 2, and domain of sequence homology or divergence amongst the family, such as those that can readily be identified using protein alignment methods and as presented in the Figures.

Antibodies are preferably prepared from regions or discrete fragments of the kinase proteins. Antibodies can be prepared from any region of the peptide as described herein. However, preferred regions will include those involved in function/activity and/or kinase/binding partner interaction. FIG. 2 can be used to identify particularly important regions while sequence alignment can be used to identify conserved and unique sequence fragments.

An antigenic fragment will typically comprise at least 8 contiguous amino acid residues. The antigenic peptide can comprise, however, at least 10, 12, 14, 16 or more amino acid residues. Such fragments can be selected on a physical property, such as fragments correspond to regions that are located on the surface of the protein, e.g., hydrophilic regions or can be selected based on sequence uniqueness (see FIG. 2).

Detection on an antibody of the present invention can be facilitated by coupling (i.e., physically linking) the antibody

to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

Antibody Uses

The antibodies can be used to isolate one of the proteins of the present invention by standard techniques, such as affinity chromatography or immunoprecipitation. The antibodies can facilitate the purification of the natural protein from cells and recombinantly produced protein expressed in host cells. In addition, such antibodies are useful to detect the presence of one of the proteins of the present invention in cells or tissues to determine the pattern of expression of the protein among various tissues in an organism and over the course of normal development. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain. Further, such antibodies can be used to detect protein in situ, in vitro, or in a cell lysate or supernatant in order to evaluate the abundance and pattern of expression. Also, such antibodies can be used to assess abnormal tissue distribution or abnormal expression during development or progression of a biological condition. Antibody detection of circulating fragments of the full length protein can be used to identify turnover.

Further, the antibodies can be used to assess expression in disease states such as in active stages of the disease or in an individual with a predisposition toward disease related to the protein's function. When a disorder is caused by an inappropriate tissue distribution, developmental expression, level of expression of the protein, or expressed/processed form, the antibody can be prepared against the normal protein. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. If a disorder is characterized by a specific mutation in the protein, antibodies specific for this mutant protein can be used to assay for the presence of the specific mutant protein.

The antibodies can also be used to assess normal and aberrant subcellular localization of cells in the various tissues in an organism. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. The diagnostic uses can be applied, not only in genetic testing, but also in monitoring a treatment modality. Accordingly, where treatment is ultimately aimed at correcting expression level or the presence of aberrant sequence and aberrant tissue distribution or developmental expression, antibodies directed against the protein or relevant fragments can be used to monitor therapeutic efficacy. Additionally, antibodies are useful in pharmacogenomic analysis. Thus, antibodies prepared against polymorphic

proteins can be used to identify individuals that require modified treatment modalities. The antibodies are also useful as diagnostic tools as an immunological marker for aberrant protein analyzed by electrophoretic mobility, isoelectric point, tryptic peptide digest, and other physical assays known to those in the art.

The antibodies are also useful for tissue typing. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. Thus, where a specific protein has been correlated with expression in a specific tissue, antibodies that are specific for this protein can be used to identify a tissue type.

The antibodies are also useful for inhibiting protein function, for example, blocking the binding of the kinase peptide to a binding partner such as a substrate. These uses can also be applied in a therapeutic context in which treatment involves inhibiting the protein's function. An antibody can be used, for example, to block binding, thus modulating (agonizing or antagonizing) the peptides activity. Antibodies can be prepared against specific fragments containing sites required for function or against intact protein that is associated with a cell or cell membrane. See FIG. 2 for structural information relating to the proteins of the present invention.

The invention also encompasses kits for using antibodies to detect the presence of a protein in a biological sample. The kit can comprise antibodies such as a labeled or labelable antibody and a compound or agent for detecting protein in a biological sample; means for determining the amount of protein in the sample; means for comparing the amount of protein in the sample with a standard; and instructions for use. Such a kit can be supplied to detect a single protein or epitope or can be configured to detect one of a multitude of epitopes, such as in an antibody detection array. Arrays are described in detail below for nucleic acid arrays and similar methods have been developed for antibody arrays.

Nucleic Acid Molecules

The present invention further provides isolated nucleic acid molecules that encode a kinase peptide or protein of the present invention (cDNA, transcript and genomic sequence). Such nucleic acid molecules will consist of, consist essentially of, or comprise a nucleotide sequence that encodes one of the kinase peptides of the present invention, an allelic variant thereof, or an ortholog or paralog thereof.

As used herein, an "isolated" nucleic acid molecule is one that is separated from other nucleic acid present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. However, there can be some flanking nucleotide sequences, for example up to about 5KB, 4KB, 3KB, 2KB, or 1KB or less, particularly contiguous peptide encoding sequences and peptide encoding sequences within the same gene but separated by introns in the genomic sequence. The important point is that the nucleic acid is isolated from remote and unimportant flanking sequences such that it can be subjected to the specific manipulations described herein such as recombinant expression, preparation of probes and primers, and other uses specific to the nucleic acid sequences.

Moreover, an "isolated" nucleic acid molecule, such as a transcript/cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by

recombinant techniques, or chemical precursors or other chemicals when chemically synthesized. However, the nucleic acid molecule can be fused to other coding or regulatory sequences and still be considered isolated.

For example, recombinant DNA molecules contained in a vector are considered isolated. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the isolated DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Accordingly, the present invention provides nucleic acid molecules that consist of the nucleotide sequence shown in FIG. 1 or 3 (SEQ ID NO:1, transcript sequence and SEQ ID NO:3, genomic sequence), or any nucleic acid molecule that encodes the protein provided in FIG. 2, SEQ ID NO:2. A nucleic acid molecule consists of a nucleotide sequence when the nucleotide sequence is the complete nucleotide sequence of the nucleic acid molecule.

The present invention further provides nucleic acid molecules that consist essentially of the nucleotide sequence shown in FIG. 1 or 3 (SEQ ID NO:1, transcript sequence and SEQ ID NO:3, genomic sequence), or any nucleic acid molecule that encodes the protein provided in FIG. 2, SEQ ID NO:2. A nucleic acid molecule consists essentially of a nucleotide sequence when such a nucleotide sequence is present with only a few additional nucleic acid residues in the final nucleic acid molecule.

The present invention further provides nucleic acid molecules that comprise the nucleotide sequences shown in FIG. 1 or 3 (SEQ ID NO:1, transcript sequence and SEQ ID NO:3, genomic sequence), or any nucleic acid molecule that encodes the protein provided in FIG. 2, SEQ ID NO:2. A nucleic acid molecule comprises a nucleotide sequence when the nucleotide sequence is at least part of the final nucleotide sequence of the nucleic acid molecule. In such a fashion, the nucleic acid molecule can be only the nucleotide sequence or have additional nucleic acid residues, such as nucleic acid residues that are naturally associated with it or heterologous nucleotide sequences. Such a nucleic acid molecule can have a few additional nucleotides or can comprise several hundred or more additional nucleotides. A brief description of how various types of these nucleic acid molecules can be readily made/isolated is provided below.

In FIGS. 1 and 3, both coding and non-coding sequences are provided. Because of the source of the present invention, humans genomic sequence (FIG. 3) and cDNA/transcript sequences (FIG. 1), the nucleic acid molecules in the Figures will contain genomic intronic sequences, 5' and 3' non-coding sequences, gene regulatory regions and non-coding intergenic sequences. In general such sequence features are either noted in FIGS. 1 and 3 or can readily be identified using computational tools known in the art. As discussed below, some of the non-coding regions, particularly gene regulatory elements such as promoters, are useful for a variety of purposes, e.g. control of heterologous gene expression, target for identifying gene activity modulating compounds, and are particularly claimed as fragments of the genomic sequence provided herein.

The isolated nucleic acid molecules can encode the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature peptide (when the mature form has more than one peptide chain, for instance). Such sequences may play a role in processing of

a protein from precursor to a mature form, facilitate protein trafficking, prolong or shorten protein half-life or facilitate manipulation of a protein for assay or production, among other things. As generally is the case in situ, the additional amino acids may be processed away from the mature protein by cellular enzymes.

As mentioned above, the isolated nucleic acid molecules include, but are not limited to, the sequence encoding the kinase peptide alone, the sequence encoding the mature peptide and additional coding sequences, such as a leader or secretory sequence (e.g., a pre-pro or pro-protein sequence), the sequence encoding the mature peptide, with or without the additional coding sequences, plus additional non-coding sequences, for example introns and non-coding 5' and 3' sequences such as transcribed but non-translated sequences that play a role in transcription, mRNA processing (including splicing and polyadenylation signals), ribosome binding and stability of mRNA. In addition, the nucleic acid molecule may be fused to a marker sequence encoding, for example, a peptide that facilitates purification.

Isolated nucleic acid molecules can be in the form of RNA, such as mRNA, or in the form DNA, including cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The nucleic acid, especially DNA, can be double-stranded or single-stranded. Single-stranded nucleic acid can be the coding strand (sense strand) or the non-coding strand (anti-sense strand).

The invention further provides nucleic acid molecules that encode fragments of the peptides of the present invention as well as nucleic acid molecules that encode obvious variants of the kinase proteins of the present invention that are described above. Such nucleic acid molecules may be naturally occurring, such as allelic variants (same locus), paralogs (different locus), and orthologs (different organism), or may be constructed by recombinant DNA methods or by chemical synthesis. Such non-naturally occurring variants may be made by mutagenesis techniques, including those applied to nucleic acid molecules, cells, or organisms. Accordingly, as discussed above, the variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions.

The present invention further provides non-coding fragments of the nucleic acid molecules provided in FIGS. 1 and 3. Preferred non-coding fragments include, but are not limited to, promoter sequences, enhancer sequences, gene modulating sequences and gene termination sequences. Such fragments are useful in controlling heterologous gene expression and in developing screens to identify gene-modulating agents. A promoter can readily be identified as being 5' to the ATG start site in the genomic sequence provided in FIG. 3.

A fragment comprises a contiguous nucleotide sequence greater than 12 or more nucleotides. Further, a fragment could at least 30, 40, 50, 100, 250 or 500 nucleotides in length. The length of the fragment will be based on its intended use. For example, the fragment can encode epitope bearing regions of the peptide, or can be useful as DNA probes and primers. Such fragments can be isolated using the known nucleotide sequence to synthesize an oligonucleotide probe. A labeled probe can then be used to screen a cDNA library, genomic DNA library, or mRNA to isolate nucleic acid corresponding to the coding region. Further, primers can be used in PCR reactions to clone specific regions of gene.

A probe/primer typically comprises substantially a purified oligonucleotide or oligonucleotide pair. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, 20, 25, 40, 50 or more consecutive nucleotides.

Orthologs, homologs, and allelic variants can be identified using methods well known in the art. As described in the Peptide Section, these variants comprise a nucleotide sequence encoding a peptide that is typically 60–70%, 70–80%, 80–90%, and more typically at least about 90–95% or more homologous to the nucleotide sequence shown in the Figure sheets or a fragment of this sequence. Such nucleic acid molecules can readily be identified as being able to hybridize under moderate to stringent conditions, to the nucleotide sequence shown in the Figure sheets or a fragment of the sequence. Allelic variants can readily be determined by genetic locus of the encoding gene. The gene encoding the novel kinase protein of the present invention is located on a genome component that has been mapped to human chromosome 22 (as indicated in FIG. 3), which is supported by multiple lines of evidence, such as STS and BAC map data.

FIG. 3 provides information on SNPs that have been found in the gene encoding the kinase protein of the present invention. SNPs were identified at 42 different nucleotide positions. Some of these SNPs, which are located outside the ORF and in introns, may affect gene transcription.

As used herein, the term “hybridizes under stringent conditions” is intended to describe conditions for hybridization and washing under which nucleotide sequences encoding a peptide at least 60–70% homologous to each other typically remain hybridized to each other. The conditions can be such that sequences at least about 60%, at least about 70%, or at least about 80% or more homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1–6.3.6. One example of stringent hybridization conditions are hybridization in 6x sodium chloride/sodium citrate (SSC) at about 45C, followed by one or more washes in 0.2xSSC, 0.1% SDS at 50–65C. Examples of moderate to low stringency hybridization conditions are well known in the art.

Nucleic Acid Molecule Uses

The nucleic acid molecules of the present invention are useful for probes, primers, chemical intermediates, and in biological assays. The nucleic acid molecules are useful as a hybridization probe for messenger RNA, transcript/cDNA and genomic DNA to isolate full-length cDNA and genomic clones encoding the peptide described in FIG. 2 and to isolate cDNA and genomic clones that correspond to variants (alleles, orthologs, etc.) producing the same or related peptides shown in FIG. 2. As illustrated in FIG. 3, SNPs were identified at 42 different nucleotide positions.

The probe can correspond to any sequence along the entire length of the nucleic acid molecules provided in the Figures. Accordingly, it could be derived from 5' noncoding regions, the coding region, and 3' noncoding regions. However, as discussed, fragments are not to be construed as encompassing fragments disclosed prior to the present invention.

The nucleic acid molecules are also useful as primers for PCR to amplify any given region of a nucleic acid molecule and are useful to synthesize antisense molecules of desired length and sequence.

The nucleic acid molecules are also useful for constructing recombinant vectors. Such vectors include expression vectors that express a portion of, or all of, the peptide sequences. Vectors also include insertion vectors, used to integrate into another nucleic acid molecule sequence, such as into the cellular genome, to alter in situ expression of a gene and/or gene product. For example, an endogenous coding sequence can be replaced via homologous recombination with all or part of the coding region containing one or more specifically introduced mutations.

The nucleic acid molecules are also useful for expressing antigenic portions of the proteins.

The nucleic acid molecules are also useful as probes for determining the chromosomal positions of the nucleic acid molecules by means of in situ hybridization methods. The gene encoding the novel kinase protein of the present invention is located on a genome component that has been mapped to human chromosome 22 (as indicated in FIG. 3), which is supported by multiple lines of evidence, such as STS and BAC map data.

The nucleic acid molecules are also useful in making vectors containing the gene regulatory regions of the nucleic acid molecules of the present invention.

The nucleic acid molecules are also useful for designing ribozymes corresponding to all, or a part, of the mRNA produced from the nucleic acid molecules described herein.

The nucleic acid molecules are also useful for making vectors that express part, or all, of the peptides.

The nucleic acid molecules are also useful for constructing host cells expressing a part, or all, of the nucleic acid molecules and peptides.

The nucleic acid molecules are also useful for constructing transgenic animals expressing all, or a part, of the nucleic acid molecules and peptides.

The nucleic acid molecules are also useful as hybridization probes for determining the presence, level, form and distribution of nucleic acid expression. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain. Accordingly, the probes can be used to detect the presence of, or to determine levels of, a specific nucleic acid molecule in cells, tissues, and in organisms. The nucleic acid whose level is determined can be DNA or RNA. Accordingly, probes corresponding to the peptides described herein can be used to assess expression and/or gene copy number in a given cell, tissue, or organism. These uses are relevant for diagnosis of disorders involving an increase or decrease in kinase protein expression relative to normal results.

In vitro techniques for detection of mRNA include Northern hybridizations and in situ hybridizations. In vitro techniques for detecting DNA includes Southern hybridizations and in situ hybridization.

Probes can be used as a part of a diagnostic test kit for identifying cells or tissues that express a kinase protein, such as by measuring a level of a kinase-encoding nucleic acid in a sample of cells from a subject e.g., mRNA or genomic DNA, or determining if a kinase gene has been mutated. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by

virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain.

Nucleic acid expression assays are useful for drug screening to identify compounds that modulate kinase nucleic acid expression.

The invention thus provides a method for identifying a compound that can be used to treat a disorder associated with nucleic acid expression of the kinase gene, particularly biological and pathological processes that are mediated by the kinase in cells and tissues that express it. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland. The method typically includes assaying the ability of the compound to modulate the expression of the kinase nucleic acid and thus identifying a compound that can be used to treat a disorder characterized by undesired kinase nucleic acid expression. The assays can be performed in cell-based and cell-free systems. Cell-based assays include cells naturally expressing the kinase nucleic acid or recombinant cells genetically engineered to express specific nucleic acid sequences.

The assay for kinase nucleic acid expression can involve direct assay of nucleic acid levels, such as mRNA levels, or on collateral compounds involved in the signal pathway. Further, the expression of genes that are up- or down-regulated in response to the kinase protein signal pathway can also be assayed. In this embodiment the regulatory regions of these genes can be operably linked to a reporter gene such as luciferase.

Thus, modulators of kinase gene expression can be identified in a method wherein a cell is contacted with a candidate compound and the expression of mRNA determined. The level of expression of kinase mRNA in the presence of the candidate compound is compared to the level of expression of kinase mRNA in the absence of the candidate compound. The candidate compound can then be identified as a modulator of nucleic acid expression based on this comparison and be used, for example to treat a disorder characterized by aberrant nucleic acid expression. When expression of mRNA is statistically significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of nucleic acid expression. When nucleic acid expression is statistically significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of nucleic acid expression.

The invention further provides methods of treatment, with the nucleic acid as a target, using a compound identified through drug screening as a gene modulator to modulate kinase nucleic acid expression in cells and tissues that express the kinase. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain. Modulation includes both up-regulation (i.e. activation or agonization) or down-regulation (suppression or antagonization) or nucleic acid expression.

Alternatively, a modulator for kinase nucleic acid expression can be a small molecule or drug identified using the screening assays described herein as long as the drug or small molecule inhibits the kinase nucleic acid expression in the cells and tissues that express the protein. Experimental data as provided in FIG. 1 indicates expression in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant and fetal brain, and thyroid gland.

The nucleic acid molecules are also useful for monitoring the effectiveness of modulating compounds on the expression or activity of the kinase gene in clinical trials or in a treatment regimen. Thus, the gene expression pattern can serve as a barometer for the continuing effectiveness of treatment with the compound, particularly with compounds to which a patient can develop resistance. The gene expression pattern can also serve as a marker indicative of a physiological response of the affected cells to the compound. Accordingly, such monitoring would allow either increased administration of the compound or the administration of alternative compounds to which the patient has not become resistant. Similarly, if the level of nucleic acid expression falls below a desirable level, administration of the compound could be commensurately decreased.

The nucleic acid molecules are also useful in diagnostic assays for qualitative changes in kinase nucleic acid expression, and particularly in qualitative changes that lead to pathology. The nucleic acid molecules can be used to detect mutations in kinase genes and gene expression products such as mRNA. The nucleic acid molecules can be used as hybridization probes to detect naturally occurring genetic mutations in the kinase gene and thereby to determine whether a subject with the mutation is at risk for a disorder caused by the mutation. Mutations include deletion, addition, or substitution of one or more nucleotides in the gene, chromosomal rearrangement, such as inversion or transposition, modification of genomic DNA, such as aberrant methylation patterns or changes in gene copy number, such as amplification. Detection of a mutated form of the kinase gene associated with a dysfunction provides a diagnostic tool for an active disease or susceptibility to disease when the disease results from overexpression, underexpression, or altered expression of a kinase protein.

Individuals carrying mutations in the kinase gene can be detected at the nucleic acid level by a variety of techniques. FIG. 3 provides information on SNPs that have been found in the gene encoding the kinase protein of the present invention. SNPs were identified at 42 different nucleotide positions. Some of these SNPs, which are located outside the ORF and in introns, may affect gene transcription. The gene encoding the novel kinase protein of the present invention is located on a genome component that has been mapped to human chromosome 22 (as indicated in FIG. 3), which is supported by multiple lines of evidence, such as STS and BAC map data. Genomic DNA can be analyzed directly or can be amplified by using PCR prior to analysis. RNA or cDNA can be used in the same way. In some uses, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., *Science* 241:1077-1080 (1988); and Nakazawa et al., *PNAS* 91:360-364 (1994)), the latter of which can be particularly useful for detecting point mutations in the gene (see Abravaya et al., *Nucleic Acids Res.* 23:675-682 (1995)). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a gene under conditions such that hybridization and amplification of the gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. Deletions and insertions can be detected by a change in size of the amplified product compared to the normal

genotype. Point mutations can be identified by hybridizing amplified DNA to normal RNA or antisense DNA sequences.

Alternatively, mutations in a kinase gene can be directly identified, for example, by alterations in restriction enzyme digestion patterns determined by gel electrophoresis.

Further, sequence-specific ribozymes (U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site. Perfectly matched sequences can be distinguished from mismatched sequences by nuclease cleavage digestion assays or by differences in melting temperature.

Sequence changes at specific locations can also be assessed by nuclease protection assays such as RNase and S1 protection or the chemical cleavage method. Furthermore, sequence differences between a mutant kinase gene and a wild-type gene can be determined by direct DNA sequencing. A variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve, C. W., (1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen et al., *Adv. Chromatogr.* 36:127-162 (1996); and Griffin et al., *Appl. Biochem. Biotechnol.* 38:147-159 (1993)).

Other methods for detecting mutations in the gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., *Science* 230:1242 (1985)); Cotton et al., *PNAS* 85:4397 (1988); Saleeba et al., *Meth. Enzymol.* 217:286-295 (1992)), electrophoretic mobility of mutant and wild type nucleic acid is compared (Orita et al., *PNAS* 86:2766 (1989); Cotton et al., *Mutat. Res.* 285:125-144 (1993); and Hayashi et al., *Genet. Anal. Tech. Appl.* 9:73-79 (1992)), and movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (Myers et al., *Nature* 313:495 (1985)). Examples of other techniques for detecting point mutations include selective oligonucleotide hybridization, selective amplification, and selective primer extension.

The nucleic acid molecules are also useful for testing an individual for a genotype that while not necessarily causing the disease, nevertheless affects the treatment modality. Thus, the nucleic acid molecules can be used to study the relationship between an individual's genotype and the individual's response to a compound used for treatment (pharmacogenomic relationship). Accordingly, the nucleic acid molecules described herein can be used to assess the mutation content of the kinase gene in an individual in order to select an appropriate compound or dosage regimen for treatment. FIG. 3 provides information on SNPs that have been found in the gene encoding the kinase protein of the present invention. SNPs were identified at 42 different nucleotide positions. Some of these SNPs, which are located outside the ORF and in introns, may affect gene transcription.

Thus nucleic acid molecules displaying genetic variations that affect treatment provide a diagnostic target that can be used to tailor treatment in an individual. Accordingly, the production of recombinant cells and animals containing these polymorphisms allow effective clinical design of treatment compounds and dosage regimens.

The nucleic acid molecules are thus useful as antisense constructs to control kinase gene expression in cells, tissues, and organisms. A DNA antisense nucleic acid molecule is designed to be complementary to a region of the gene

involved in transcription, preventing transcription and hence production of kinase protein. An antisense RNA or DNA nucleic acid molecule would hybridize to the mRNA and thus block translation of mRNA into kinase protein.

Alternatively, a class of antisense molecules can be used to inactivate mRNA in order to decrease expression of kinase nucleic acid. Accordingly, these molecules can treat a disorder characterized by abnormal or undesired kinase nucleic acid expression. This technique involves cleavage by means of ribozymes containing nucleotide sequences complementary to one or more regions in the mRNA that attenuate the ability of the mRNA to be translated. Possible regions include coding regions and particularly coding regions corresponding to the catalytic and other functional activities of the kinase protein, such as substrate binding.

The nucleic acid molecules also provide vectors for gene therapy in patients containing cells that are aberrant in kinase gene expression. Thus, recombinant cells, which include the patient's cells that have been engineered ex vivo and returned to the patient, are introduced into an individual where the cells produce the desired kinase protein to treat the individual.

The invention also encompasses kits for detecting the presence of a kinase nucleic acid in a biological sample. Experimental data as provided in FIG. 1 indicates that the kinase proteins of the present invention are expressed in humans in teratocarcinoma, ovary, testis, nervous tissue, bladder, infant brain, and thyroid gland, as indicated by virtual northern blot analysis. In addition, PCR-based tissue screening panels indicate expression in fetal brain. For example, the kit can comprise reagents such as a labeled or labelable nucleic acid or agent capable of detecting kinase nucleic acid in a biological sample; means for determining the amount of kinase nucleic acid in the sample; and means for comparing the amount of kinase nucleic acid in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect kinase protein mRNA or DNA.

Nucleic Acid Arrays

The present invention further provides nucleic acid detection kits, such as arrays or microarrays of nucleic acid molecules that are based on the sequence information provided in FIGS. 1 and 3 (SEQ ID NOS:1 and 3).

As used herein "Arrays" or "Microarrays" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support. In one embodiment, the microarray is prepared and used according to the methods described in U.S. Pat. No. 5,837,832, Chee et al., PCT application W095/11995 (Chee et al.), Lockhart, D. J. et al. (1996; *Nat. Biotech.* 14: 1675-1680) and Schena, M. et al. (1996; *Proc. Natl. Acad. Sci.* 93: 10614-10619), all of which are incorporated herein in their entirety by reference. In other embodiments, such arrays are produced by the methods described by Brown et al., U.S. Pat. No. 5,807,522.

The microarray or detection kit is preferably composed of a large number of unique, single-stranded nucleic acid sequences, usually either synthetic antisense oligonucleotides or fragments of cDNAs, fixed to a solid support. The oligonucleotides are preferably about 6-60 nucleotides in length, more preferably 15-30 nucleotides in length, and most preferably about 20-25 nucleotides in length. For a certain type of microarray or detection kit, it may be

preferable to use oligonucleotides that are only 7–20 nucleotides in length. The microarray or detection kit may contain oligonucleotides that cover the known 5', or 3', sequence, sequential oligonucleotides which cover the full length sequence; or unique oligonucleotides selected from particular areas along the length of the sequence. Polynucleotides used in the microarray or detection kit may be oligonucleotides that are specific to a gene or genes of interest.

In order to produce oligonucleotides to a known sequence for a microarray or detection kit, the gene(s) of interest (or an ORF identified from the contigs of the present invention) is typically examined using a computer algorithm which starts at the 5' or at the 3' end of the nucleotide sequence. Typical algorithms will then identify oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. In certain situations it may be appropriate to use pairs of oligonucleotides on a microarray or detection kit. The "pairs" will be identical, except for one nucleotide that preferably is located in the center of the sequence. The second oligonucleotide in the pair (mismatched by one) serves as a control. The number of oligonucleotide pairs may range from two to one million. The oligomers are synthesized at designated areas on a substrate using a light-directed chemical process. The substrate may be paper, nylon or other type of membrane, filter, chip, glass slide or any other suitable solid support.

In another aspect, an oligonucleotide may be synthesized on the surface of the substrate by using a chemical coupling procedure and an ink jet application apparatus, as described in PCT application W095/251116 (Balteschweiler et al.) which is incorporated herein in its entirety by reference. In another aspect, a "gridded" array analogous to a dot (or slot) blot may be used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. An array, such as those described above, may be produced by hand or by using available devices (slot blot or dot blot apparatus), materials (any suitable solid support), and machines (including robotic instruments), and may contain 8, 24, 96, 384, 1536, 6144 or more oligonucleotides, or any other number between two and one million which lends itself to the efficient use of commercially available instrumentation.

In order to conduct sample analysis using a microarray or detection kit, the RNA or DNA from a biological sample is made into hybridization probes. The mRNA is isolated, and cDNA is produced and used as a template to make antisense RNA (aRNA). The aRNA is amplified in the presence of fluorescent nucleotides, and labeled probes are incubated with the microarray or detection kit so that the probe sequences hybridize to complementary oligonucleotides of the microarray or detection kit. Incubation conditions are adjusted so that hybridization occurs with precise complementary matches or with various degrees of less complementarity. After removal of nonhybridized probes, a scanner is used to determine the levels and patterns of fluorescence. The scanned images are examined to determine degree of complementarity and the relative abundance of each oligonucleotide sequence on the microarray or detection kit. The biological samples may be obtained from any bodily fluids (such as blood, urine, saliva, phlegm, gastric juices, etc.), cultured cells, biopsies, or other tissue preparations. A detection system may be used to measure the absence, presence, and amount of hybridization for all of the distinct sequences simultaneously. This data may be used for large-scale correlation studies on the sequences, expression patterns, mutations, variants, or polymorphisms among samples.

Using such arrays, the present invention provides methods to identify the expression of the kinase proteins/peptides of the present invention. In detail, such methods comprise incubating a test sample with one or more nucleic acid molecules and assaying for binding of the nucleic acid molecule with components within the test sample. Such assays will typically involve arrays comprising many genes, at least one of which is a gene of the present invention or alleles of the kinase gene of the present invention. FIG. 3 provides information on SNPs that have been found in the gene encoding the kinase protein of the present invention. SNPs were identified at 42 different nucleotide positions. Some of these SNPs, which are located outside the ORF and in introns, may affect gene transcription.

Conditions for incubating a nucleic acid molecule with a test sample vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the nucleic acid molecule used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or array assay formats can readily be adapted to employ the novel fragments of the Human genome disclosed herein. Examples of such assays can be found in Chard, T, *An Introduction to Radioimmunoassay and Related Techniques*, Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock, G. R. et al., *Techniques in Immunocytochemistry*, Academic Press, Orlando, Fla. Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., *Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The test samples of the present invention include cells, protein or membrane extracts of cells. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing nucleic acid extracts or of cells are well known in the art and can be readily be adapted in order to obtain a sample that is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention.

Specifically, the invention provides a compartmentalized kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the nucleic acid molecules that can bind to a fragment of the Human genome disclosed herein; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound nucleic acid.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers, strips of plastic, glass or paper, or arraying material such as silica. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the nucleic acid probe, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound probe. One skilled in the art will readily recognize that the previously unidentified kinase gene of the present invention can be routinely identified using the sequence information disclosed herein can be readily incorporated into one of the established kit formats which are well known in the art, particularly expression arrays.

The invention also provides vectors containing the nucleic acid molecules described herein. The term "vector" refers to a vehicle, preferably a nucleic acid molecule, which can transport the nucleic acid molecules. When the vector is a nucleic acid molecule, the nucleic acid molecules are covalently linked to the vector nucleic acid. With this aspect of the invention, the vector includes a plasmid, single or double stranded phage, a single or double stranded RNA or DNA viral vector, or artificial chromosome, such as a BAC, PAC, YAC, OR MAC.

A vector can be maintained in the host cell as an extra-chromosomal element where it replicates and produces additional copies of the nucleic acid molecules. Alternatively, the vector may integrate into the host cell genome and produce additional copies of the nucleic acid molecules when the host cell replicates.

The invention provides vectors for the maintenance (cloning vectors) or vectors for expression (expression vectors) of the nucleic acid molecules. The vectors can function in prokaryotic or eukaryotic cells or in both (shuttle vectors).

Expression vectors contain cis-acting regulatory regions that are operably linked in the vector to the nucleic acid molecules such that transcription of the nucleic acid molecules is allowed in a host cell. The nucleic acid molecules can be introduced into the host cell with a separate nucleic acid molecule capable of affecting transcription. Thus, the second nucleic acid molecule may provide a trans-acting factor interacting with the cis-regulatory control region to allow transcription of the nucleic acid molecules from the vector. Alternatively, a trans-acting factor may be supplied by the host cell. Finally, a trans-acting factor can be produced from the vector itself. It is understood, however, that in some embodiments, transcription and/or translation of the nucleic acid molecules can occur in a cell-free system.

The regulatory sequence to which the nucleic acid molecules described herein can be operably linked include promoters for directing mRNA transcription. These include, but are not limited to, the left promoter from bacteriophage λ , the lac, TRP, and TAC promoters from *E. coli*, the early and late promoters from SV40, the CMV immediate early promoter, the adenovirus early and late promoters, and retrovirus long-terminal repeats.

In addition to control regions that promote transcription, expression vectors may also include regions that modulate transcription, such as repressor binding sites and enhancers. Examples include the SV40 enhancer, the cytomegalovirus immediate early enhancer, polyoma enhancer, adenovirus enhancers, and retrovirus LTR enhancers.

In addition to containing sites for transcription initiation and control, expression vectors can also contain sequences necessary for transcription termination and, in the transcribed region a ribosome binding site for translation. Other regulatory control elements for expression include initiation and termination codons as well as polyadenylation signals. The person of ordinary skill in the art would be aware of the numerous regulatory sequences that are useful in expression vectors. Such regulatory sequences are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

A variety of expression vectors can be used to express a nucleic acid molecule. Such vectors include chromosomal, episomal, and virus-derived vectors, for example vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, including yeast artificial chromosomes, from viruses such as baculoviruses, papovaviruses such as SV40, Vaccinia

viruses, adenoviruses, poxviruses, pseudorabies viruses, and retroviruses. Vectors may also be derived from combinations of these sources such as those derived from plasmid and bacteriophage genetic elements, e.g. cosmids and phagemids. Appropriate cloning and expression vectors for prokaryotic and eukaryotic hosts are described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

The regulatory sequence may provide constitutive expression in one or more host cells (i.e. tissue specific) or may provide for inducible expression in one or more cell types such as by temperature, nutrient additive, or exogenous factor such as a hormone or other ligand. A variety of vectors providing for constitutive and inducible expression in prokaryotic and eukaryotic hosts are well known to those of ordinary skill in the art.

The nucleic acid molecules can be inserted into the vector nucleic acid by well-known methodology. Generally, the DNA sequence that will ultimately be expressed is joined to an expression vector by cleaving the DNA sequence and the expression vector with one or more restriction enzymes and then ligating the fragments together. Procedures for restriction enzyme digestion and ligation are well known to those of ordinary skill in the art.

The vector containing the appropriate nucleic acid molecule can be introduced into an appropriate host cell for propagation or expression using well-known techniques. Bacterial cells include, but are not limited to, *E. coli*, *Streptomyces*, and *Salmonella typhimurium*. Eukaryotic cells include, but are not limited to, yeast, insect cells such as *Drosophila*, animal cells such as COS and CHO cells, and plant cells.

As described herein, it may be desirable to express the peptide as a fusion protein. Accordingly, the invention provides fusion vectors that allow for the production of the peptides. Fusion vectors can increase the expression of a recombinant protein, increase the solubility of the recombinant protein, and aid in the purification of the protein by acting for example as a ligand for affinity purification. A proteolytic cleavage site may be introduced at the junction of the fusion moiety so that the desired peptide can ultimately be separated from the fusion moiety. Proteolytic enzymes include, but are not limited to, factor Xa, thrombin, and enterokinase. Typical fusion expression vectors include pGEX (Smith et al., *Gene* 67:31-40 (1988)), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., *Gene* 69:301-315 (1988)) and pET 11 d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185:60-89 (1990)).

Recombinant protein expression can be maximized in host bacteria by providing a genetic background wherein the host cell has an impaired capacity to proteolytically cleave the recombinant protein. (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 119-128). Alternatively, the sequence of the nucleic acid molecule of interest can be altered to provide preferential codon usage for a specific host cell, for example *E. coli*. (Wada et al., *Nucleic Acids Res.* 20:2111-2118 (1992)).

The nucleic acid molecules can also be expressed by expression vectors that are operative in yeast. Examples of vectors for expression in yeast e.g., *S. cerevisiae* include pYepSec1 (Baldari, et al., *EMBO J.* 6:229-234 (1987)), pMFa (Kurjan et al., *Cell* 30:933-943(1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, Calif.).

The nucleic acid molecules can also be expressed in insect cells using, for example, baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al., *Mol. Cell Biol.* 3:2156-2165 (1983)) and the pVL series (Lucklow et al., *Virology* 170:31-39 (1989)).

In certain embodiments of the invention, the nucleic acid molecules described herein are expressed in mammalian cells using mammalian expression vectors. Examples of mammalian expression vectors include pCDM8 (Seed, B. *Nature* 329:840(1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).

The expression vectors listed herein are provided by way of example only of the well-known vectors available to those of ordinary skill in the art that would be useful to express the nucleic acid molecules. The person of ordinary skill in the art would be aware of other vectors suitable for maintenance propagation or expression of the nucleic acid molecules described herein. These are found for example in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

The invention also encompasses vectors in which the nucleic acid sequences described herein are cloned into the vector in reverse orientation, but operably linked to a regulatory sequence that permits transcription of antisense RNA. Thus, an antisense transcript can be produced to all, or to a portion, of the nucleic acid molecule sequences described herein, including both coding and non-coding regions. Expression of this antisense RNA is subject to each of the parameters described above in relation to expression of the sense RNA (regulatory sequences, constitutive or inducible expression, tissue-specific expression).

The invention also relates to recombinant host cells containing the vectors described herein. Host cells therefore include prokaryotic cells, lower eukaryotic cells such as yeast, other eukaryotic cells such as insect cells, and higher eukaryotic cells such as mammalian cells.

The recombinant host cells are prepared by introducing the vector constructs described herein into the cells by techniques readily available to the person of ordinary skill in the art. These include, but are not limited to, calcium phosphate transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, lipofection, and other techniques such as those found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*. 2nd, ed, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

Host cells can contain more than one vector. Thus, different nucleotide sequences can be introduced on different vectors of the same cell. Similarly, the nucleic acid molecules can be introduced either alone or with other nucleic acid molecules that are not related to the nucleic acid molecules such as those providing trans-acting factors for expression vectors. When more than one vector is introduced into a cell, the vectors can be introduced independently, co-introduced or joined to the nucleic acid molecule vector.

In the case of bacteriophage and viral vectors, these can be introduced into cells as packaged or encapsulated virus by standard procedures for infection and transduction. Viral vectors can be replication-competent or replication-defective. In the case in which viral replication is defective, replication will occur in host cells providing functions that complement the defects.

Vectors generally include selectable markers that enable the selection of the subpopulation of cells that contain the

recombinant vector constructs. The marker can be contained in the same vector that contains the nucleic acid molecules described herein or may be on a separate vector. Markers include tetracycline or ampicillin-resistance genes for prokaryotic host cells and dihydrofolate reductase or neomycin resistance for eukaryotic host cells. However, any marker that provides selection for a phenotypic trait will be effective.

While the mature proteins can be produced in bacteria, yeast, mammalian cells, and other cells under the control of the appropriate regulatory sequences, cell-free transcription and translation systems can also be used to produce these proteins using RNA derived from the DNA constructs described herein.

Where secretion of the peptide is desired, which is difficult to achieve with multi-transmembrane domain containing proteins such as kinases, appropriate secretion signals are incorporated into the vector. The signal sequence can be endogenous to the peptides or heterologous to these peptides.

Where the peptide is not secreted into the medium, which is typically the case with kinases, the protein can be isolated from the host cell by standard disruption procedures, including freeze thaw, sonication, mechanical disruption, use of lysing agents and the like. The peptide can then be recovered and purified by well-known purification methods including ammonium sulfate precipitation, acid extraction, anion or cationic exchange chromatography, phosphocellulose chromatography, hydrophobic-interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, or high performance liquid chromatography.

It is also understood that depending upon the host cell in recombinant production of the peptides described herein, the peptides can have various glycosylation patterns, depending upon the cell, or maybe non-glycosylated as when produced in bacteria. In addition, the peptides may include an initial modified methionine in some cases as a result of a host-mediated process.

Uses of Vectors and Host Cells

The recombinant host cells expressing the peptides described herein have a variety of uses. First, the cells are useful for producing a kinase protein or peptide that can be further purified to produce desired amounts of kinase protein or fragments. Thus, host cells containing expression vectors are useful for peptide production.

Host cells are also useful for conducting cell-based assays involving the kinase protein or kinase protein fragments, such as those described above as well as other formats known in the art. Thus, a recombinant host cell expressing a native kinase protein is useful for assaying compounds that stimulate or inhibit kinase protein function.

Host cells are also useful for identifying kinase protein mutants in which these functions are affected. If the mutants naturally occur and give rise to a pathology, host cells containing the mutations are useful to assay compounds that have a desired effect on the mutant kinase protein (for example, stimulating or inhibiting function) which may not be indicated by their effect on the native kinase protein.

Genetically engineered host cells can be further used to produce non-human transgenic animals. A transgenic animal is preferably a mammal, for example a rodent, such as a rat or mouse, in which one or more of the cells of the animal include a transgene. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal in one or more cell types or tissues of the transgenic animal. These animals are useful for

studying the function of a kinase protein and identifying and evaluating modulators of kinase protein activity. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, and amphibians.

A transgenic animal can be produced by introducing nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Any of the kinase protein nucleotide sequences can be introduced as a transgene into the genome of a non-human animal, such as a mouse.

Any of the regulatory or other sequences useful in expression vectors can form part of the transgenic sequence. This includes intronic sequences and polyadenylation signals, if not already included. A tissue-specific regulatory sequence (s) can be operably linked to the transgene to direct expression of the kinase protein to particular cells.

Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al. U.S. Pat. No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the transgene in its genome and/or expression of transgenic mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene can further be bred to other transgenic animals carrying other transgenes. A transgenic animal also includes animals in which the entire animal or tissues in the animal have been produced using the homologously recombinant host cells described herein.

In another embodiment, transgenic non-human animals can be produced which contain selected systems that allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al. *PNAS* 89:6232-6236 (1992). Another example of a recombinase system is the FLP recombinase system of *S. cerevisiae* (O'Gorman et al. *Science* 251:1351-1355 (1991). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recom-

binase and a selected protein is required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al. *Nature* 385:810-813 (1997) and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G₀ phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring born of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

Transgenic animals containing recombinant cells that express the peptides described herein are useful to conduct the assays described herein in an in vivo context. Accordingly, the various physiological factors that are present in vivo and that could effect substrate binding, kinase protein activation, and signal transduction, may not be evident from in vitro cell-free or cell-based assays. Accordingly, it is useful to provide non-human transgenic animals to assay in vivo kinase protein function, including substrate interaction, the effect of specific mutant kinase proteins on kinase protein function and substrate interaction, and the effect of chimeric kinase proteins. It is also possible to assess the effect of null mutations, that is, mutations that substantially or completely eliminate one or more kinase protein functions.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1

<211> LENGTH: 2320

<212> TYPE: DNA

<213> ORGANISM: Human

<400> SEQUENCE: 1

```

cccaggggcgc cgtaggcgggt gcataccggtt cgcgcctggg gctgtggtct tcccgcgctt    60
gaggcggcgcg cggcaggagc tgaggggagt tgtagggaac tgaggggagc tgctgtgtcc    120
ccgcctcctcct cctccccatt tccgcgtccc cgggaccatg tccgcgctgg cgggtgaaga    180
tgtctggagg tgctcaggct gtggggacca cattgtccca agccagatat ggtacaggac    240

```


-continued

tgtaacgaa acctggcacg gctcttgctt ccggtgaaag tgatgcgcag cctggaccac	300
cccaatgtgc tcaagttcat tgggtgtgctg tacaaggata agaagctgaa cctgctgaca	360
gagtacattg agggggggcac actgaaggac tttctgcgca gtatggatcc gttcccctgg	420
cagcagaagg tcaggtttgc caaaggaatc gcctccggaa tggacaagac tgtggtggtg	480
gcagactttg ggctgtcacg gctcatagtg gaagagagga aaagggcccc catggagaag	540
gccaccacca agaaacgcac ctgctgcaag aacgaccgca agaagcgcta cagggtggtg	600
ggaaaccctt actggatggc ccttgagatg ctgaacggaa agagctatga tgagacggtg	660
gatattctct cctttgggat cgttctctgt gagatcattg ggcagggtga tgcagatcct	720
gactgccttc cccgaacact ggactttggc ctcaacgtga agcttttctg ggagaagttt	780
gttcccacag attgtcccc ggccttcttc ccgctggcgg ccactctgctg cagactggag	840
cctgagagca gaccagcatt ctcgaaattg gaggactcct ttgaggccct ctccctgtac	900
ctggggggagc tgggcatccc gctgcctgca gagctggagg agttggacca cactgtgagc	960
atgcagtacg gcctgaccgg ggaactcacct ccctagccct ggcccagccc cctgcagggg	1020
ggtgtttctac agccagcatt gccctctctg gccccattcc tgctgtgagc agggccgtcc	1080
gggcttctctg tggattggcg gaattgttag aagcagaaca aaccattcct attacctccc	1140
caggaggcaa gtgggcgcag caccagggaa atgtatctcc acaggttctg gggcctagtt	1200
actgtctgta aatccaatac ttgcctgaaa gctgtgaaga agaaaaaac ccctggcctt	1260
tgggccagga ggaatctgtt actcgaatcc acccaggaac tccctggcag tggattgtgg	1320
gaggctcttg cttacactaa tcagcgtgac ctggacotgc tgggcaggat cccaggggtga	1380
acctgcctgt gaactctgaa gtcactagtc cagctgggtg caggaggact tcaagtgtgt	1440
ggacgaaga aagactgatg gctcaagggt tgtgaaaag tcagtgatgc tcccccttc	1500
tactccagat cctgtccttc ctggagcaag gttgaggag taggttttga agagtccctt	1560
aatatgtggt ggaacaggcc aggagttaga gaaagggtg gcttctgttt acctgctcac	1620
tggctctagc cagcccaggg accacatcaa tgtgagagga agcctccacc tcatgttttc	1680
aaacttaata ctggagactg gctgagaact tacggacaac atcctttctg totgaaacaa	1740
acagtacaaa gcacaggaag aggctggggg actagaaaga ggcctgccc tctagaaagc	1800
tcagatcttg gcttctgtta ctccatactcg ggtgggctcc ttagtcatg gcctaaaaca	1860
ttttgcctaa agctcgtatg gttctggagg acagtgtggc ttgtcacagg cctagagtct	1920
gaggaggagg agtgggagtc tcagcaatct cttgggtctt gcttcattgg aacctgct	1980
caccttcaa catgcctggt ttaggcagca gcttgggctg ggaagagggt gtggcagagt	2040
ctcaaagctg agatgctgag agagatagct ccctgagctg ggccatctga cttctacctc	2100
ccatgtttgc tctcccaact cattagctcc tgggcagcat cctcctgagc cacatgtgca	2160
ggtactggaa aacctccatc ttggctccca gagctctagg aactcttcac cacaactaga	2220
tttgctctct ctaagtgtct atgagcttgc accatattta ataaattggg aatgggtttg	2280
gggtattaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa	2320

<210> SEQ ID NO 2

<211> LENGTH: 255

<212> TYPE: PRT

<213> ORGANISM: Human

<400> SEQUENCE: 2

Met Val Gln Asp Cys Gln Arg Asn Leu Ala Arg Leu Leu Leu Pro Val

-continued

1	5	10	15
Lys Val Met Arg Ser Leu Asp His Pro Asn Val Leu Lys Phe Ile Gly	20	25	30
Val Leu Tyr Lys Asp Lys Lys Leu Asn Leu Leu Thr Glu Tyr Ile Glu	35	40	45
Gly Gly Thr Leu Lys Asp Phe Leu Arg Ser Met Asp Pro Phe Pro Trp	50	55	60
Gln Gln Lys Val Arg Phe Ala Lys Gly Ile Ala Ser Gly Met Asp Lys	65	70	75
Thr Val Val Val Ala Asp Phe Gly Leu Ser Arg Leu Ile Val Glu Glu	85	90	95
Arg Lys Arg Ala Pro Met Glu Lys Ala Thr Thr Lys Lys Arg Thr Leu	100	105	110
Arg Lys Asn Asp Arg Lys Lys Arg Tyr Thr Val Val Gly Asn Pro Tyr	115	120	125
Trp Met Ala Pro Glu Met Leu Asn Gly Lys Ser Tyr Asp Glu Thr Val	130	135	140
Asp Ile Phe Ser Phe Gly Ile Val Leu Cys Glu Ile Ile Gly Gln Val	145	150	155
Tyr Ala Asp Pro Asp Cys Leu Pro Arg Thr Leu Asp Phe Gly Leu Asn	165	170	175
Val Lys Leu Phe Trp Glu Lys Phe Val Pro Thr Asp Cys Pro Pro Ala	180	185	190
Phe Phe Pro Leu Ala Ala Ile Cys Cys Arg Leu Glu Pro Glu Ser Arg	195	200	205
Pro Ala Phe Ser Lys Leu Glu Asp Ser Phe Glu Ala Leu Ser Leu Tyr	210	215	220
Leu Gly Glu Leu Gly Ile Pro Leu Pro Ala Glu Leu Glu Glu Leu Asp	225	230	235
His Thr Val Ser Met Gln Tyr Gly Leu Thr Arg Asp Ser Pro Pro	245	250	255

<210> SEQ ID NO 3
 <211> LENGTH: 59065
 <212> TYPE: DNA
 <213> ORGANISM: Human

<400> SEQUENCE: 3

tcataccttgc gcaggggcca tgctaaccctt ctgtgtctca gtccaatttt aatgtatgtg	60
ctgtgtaagc gagagtacca gaggtttttt tgatggcagt gacttgaact tatttaaag	120
ataaggagga gccagtgagg gagaggggtg ctgtaaagat aactaaaagt gcactttctc	180
taagaagtaa gatggaatgg gatccagaac aggggtgtca taccgagtag ccagccttt	240
gttcctgtga cactggggag tctaaccag agctgagata gcttgacgtg tggatgagcc	300
agctgagtac agcagatagg gaaaagaagc caaaaatctg aagtaggggt ggggtgaagg	360
acaggggaagg gctagagaga catttgaaa gtgaaccag gtggatatga gaggagagag	420
tagaggggtc tgatttcggg tctttcatgc ttaacccaaa gcaggtacta aagtatgtgt	480
tgattgaatg tctttgggtt tctcaagact ggagaagca gggcaagctc tggaggggtat	540
ggcaataaca agttatcttg aatatactca tgggtgaaag tctgtatcct gtttgaattt	600
tggaaataga aatcattcag agccaagaga ttgaattgtt gagtaagtgg gtgggtcaggt	660
tacagactta attttgggtt aaaaagtata aacaagaac aaggtgtggc tctaaaataa	720

-continued

tgagatgtgc tgggggtggg gcatggcagc tcataaactg accctgaaag ctcttacatg	780
taagagttcc aaaaatattt ccaaaacttg gaagattcat ttggatgttt gtgttcatta	840
aaatctctca ctaattcatt gtcttgcca ctgtccgtaa cccaacctgg gattggtttg	900
agtgaagtcc tcagactttc tgccttgag tttgtgagag agatggcata ctctgtgacc	960
actgtcacc taaaacaaa aaggccctc ttgacaagga gtctgaggat ttagaccca	1020
ggaagaatga gtgatgggca tatatatc ctattactga ggcagagaa gagtggaaatg	1080
ggtgggttga ggtggtgttt taaggcctct tgccagcttg ttaactctt ctctgggaa	1140
cgagggggac aactgtgtac attggctgct ccagaatgat gttgagcaat cttgaagtgc	1200
caggagctgt gctttgtcta ttcattggcc ctgtgctgt gaaacaggg tccgtgactg	1260
tcactgtgcc tgtggcagtc tgtagttacc cagagagaac aaagctgcat acacagagcg	1320
cacaagggag tcttgaaca acctgtcct gctttctagg gctgagtcag gtaccacagc	1380
ttgatctcag ctgtcctctt tatttcaaga agttgacatc tgagccatac caggagtatt	1440
gtattttgtt tgaggcctct ctttttgag gaacatggac cgactctgtg cttttgtcta	1500
tgctggctc tgagctcaca caacccttca ccctccttc tcagccagtg ataggttaagt	1560
cttccctatc ttgcaaggct cagctcaagt gtcagcttcc tctacaaaga ctttctggt	1620
tcccctcatt ggagtgaaca agagttgaca tggtagaatg gaaagagcag aagctttaga	1680
atgagccaga cctgagtatg aatgctagat ccaccactta gctagtcaac cctgccccct	1740
gcctcaagtt ttaattttcc tatccattaa gtgaatataa taatacctgt gtcacaggat	1800
tattttgaga attaaatgag attaggtcta tgaaagcacc tagcagagtt cttggcatat	1860
aggaggcatt cattaatat ttgttcttcc ctttttatac ccattacttt tctttttctg	1920
aactaaaata atacttggtt ctatctctga aataacatcc aagtgaanaa tcaacaacat	1980
gaaagagcag ttcttttcca gtggatttgc ttcttaagga gcagagatta tgtaacttaa	2040
cagcctccaa catacaaga gctttgtatc tagaacaggg gtccccagcc cctggaccgc	2100
caactgttac ggtctctgag cctgttagga accaggctgc acagcaggag gtgagcggcg	2160
ggccagttag cattgctgcc tgagctctgc ctctgtcag atcagtgggt gcattagatt	2220
ctcataggag tgtgaacct attgtgaact gcacatgcaa gggatctggg ttgcatgctc	2280
cttatgagaa tctcactaat ggctgatgat ctgagttgga acagtttgat accaaaacca	2340
tccccccgcc ccccaacccc cagcctaggg tccgtgaaa aattggcccc tggtgccaaa	2400
aagggtgagg actgctgac tagaggacca atttattcaa tgttggttga gtaaatgagc	2460
tcttgatta ggtgatgaa aaatctgaaa aaacagggct tttgaggaat aggaaaaggc	2520
agtaacatgt ttaaccaga gagaagtctc tggctgttgg ctgggaatag tcataggaa	2580
ggctgacact gaaaagaagg agattgtgtt cgtttcttct tctcagagct ataagcaaa	2640
gctgaaagtt ctgaaaaag gcaagtttg tttcagtaga aaaaaggata atcagaacca	2700
tttttagaaa atggaatgag actactttt aggccatgag ttcctgtcc ctggagagat	2760
gagcagaggt tggacaagt ctaccagag atcttgtgga ggcagaaact gtgcactag	2820
cagagcattg gcctaacct ttcaaatgag atgctgttaa ctacgtctta ttctacatg	2880
taggaatcct gtcccttgc ctctgctac tttgggctc tcaacctctt ggttttgtgt	2940
gcagggtgaag atgtctggag gtgtccaggc tgtggggacc acattgctcc aagccagata	3000
tgttacagga ctgtcaaca aacctggcac ggctcttgct tccggtaggt gggcctatcc	3060
tcccatcttt accagtgtac tatgggcaa gcactatttc atgttctgat ggaanaacaa	3120

-continued

gaaacaagct tctgagttga gaatttcaat cttagggtgg ggaaggaat gtaccaagga	3180
agagctcatg accaaacctc aagtgtggcc cccctgaacc caggttaaat tggaagagcc	3240
ataaatgggc cagctggagg cagggtgggg ggatgagagg agccctttcc agggttgtcc	3300
catatccctc actttatggg tgaggaaact gaggccagg aagagtgaact ttctgtggc	3360
tgactacag attatgcagg tacttcaaga gttgtttgta ttctattttt attttatttt	3420
attttatttt attttatttt attttatgag agggattctt gctgttgccc aggtggagt	3480
gcagtgggc aatctcggt cactgcaatc tctgctgct gggttcaagt gatttttctg	3540
ccttagcttc ctgagtagct gagatgacag gcacctgcca ccattgcgag ctaatttttg	3600
tattttatg gagacggggg ttcaacatg ttggtcaggc tggcttgaa ctctgacct	3660
caaatgatgc acccaacctc acctcccaaa gtgctggaat tacaggcgtg aacctgtg	3720
cccagccaag agttgtttt agtgtggtg gcagagccag ctcttccttc accacaggat	3780
gcctccctag gtctcactt ttgttacta gcttttatta tagctatatt attattatta	3840
ttattattat tattattatt attattgaga cagagtctcg ctctgtcgcc caggctggtg	3900
tacagtggg cgatcccggt ctcactgcaa cctctgcctc ccgagttcaa gcagttctc	3960
tgctcagcc ccccgagtag gtgggactac aggcgcctgc caccacacce ggctaatttt	4020
tgtattttta gtagagacgg ggttcacct tgttgaccag gctggtctgg agctcctgac	4080
ctcaggtaag tgctagaatc acaggcgtga accactgcgc ccagccaaga gttgttttta	4140
gtgtggttg cagagccagc tcttcctcac cacaggttgc ctccctaggt tctactttt	4200
tggtactagc ttattatag ctacattatt attattattg ttattattat tgagacagag	4260
tctcgctcg tcgccaggc tgggtgacag tgatgtgac ttggctcact gcaacctctg	4320
ccccccagt tcaagcaatt ctctgcttc agcccccta gtaggtggga ctccaggcac	4380
ctgccaccac gccagctaa tttttgtatt tttagtagag gcggggtttc acctgttg	4440
ccaggctggt ctcaaacctc tgacctcagg tgatccgct gcctcgccct cccaaatgt	4500
tgggattaca ggcattgacc accgcgcct gcctatagct acattatttt ttaggcagc	4560
tcagtttctt aaaaattata cagacttcaa atcagatttg ttctgtctgt ctgaggctca	4620
gtttctctat ctggaaaatg gatgtaata atctgttga gattgaatga aataatatat	4680
gcagtgtatc cagtacatgg tagacacca gtgaatggtt attcttctc cccatcggt	4740
tggaattctc aagggtggga actgtctttt atattcttca caactgaaa tagttgaat	4800
ttgttggtg aaagaagagc agtccactcc agaggctgga tgggcatgcc tggccccc	4860
ggctggaat ggtagggtg tgcctatatc ctgagaatga gatagactag gcaggcacct	4920
tgtgctgtag attccagctc ctgcacatag ctctgtgtgt aaaacatccc tgtgcttata	4980
ccaagtaatt gagttgacct ttaaacactt gcctcttccc tgggaacct ataggggatt	5040
ggcctggaga cgtctgcct ctggaagagt tggaaagcag ccattcattat tacccttcc	5100
tttcagctat aactcagagc tctcaagtct ttctgtgga tcttattgcc ttggttctg	5160
ccccctttac tcccaggga gttgattctg tcttttctgt tccatttagt atgacaggag	5220
cagagaatgt cagagctgta agggacctta tagttaaac ctttggtg tcttttcat	5280
ttatagctgg gactaataag taactgcaa acccaatgag ttcacagatt ggtctcgcc	5340
ttggcatgta acccatatgt tcatattctt gctgttttcc tatgtgatg aatattttct	5400
atccaaaata agcaggacag gtagagcaa gtaaatcttt ggaatttctg gattctctta	5460

-continued

gagctaaaaa acttcagaac tagaagaac caccactat atggtataac ccattcatat	5520
cacagatgag gcctgaaacc aaaaagactt gctcaggcca tggatgacaa gagctggccc	5580
tagcactgaa ctcttgggtc attttaggt ctagtcagat gctagcttgt tagctctgtg	5640
cgtgcgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgagat agagacagaa agataacata	5700
tgtaacaaaa tacataaaga ggaagtagac acgttagcat ggtagataag agtacaggca	5760
ggccaggcgt ggtggctcac gcctgtaac ccagcacttt gggaggccaa ggcagggtga	5820
tcacctgagg tcaggaaattc gagaccagcc tgaccaacat ggtgaaaccc catctctact	5880
aaatacagaa aaaaattagc ttggcatggt ggcacatgcc tgtaatccca gctacttggg	5940
aagctgaagc aggagaatcg ctgtaatccg ggaagcagaa gttgcagtga gccgagattg	6000
tgccattaca gtctagcctg ggcaacaaga gggaaactcc atcgcaaaaa aacaaccacc	6060
accaagagta caggctatgg aatgagacta tggttttaaa tcctggcttt gcaatttatt	6120
aactagcctt aagtgaattc cctgagcttc aggcaccaat ctgtaaatg aggataagaa	6180
tattactcat gccacatggt tgttagggag gattaaatgt gataacctat ataaagtggc	6240
tagcatagca tctgacatat agaaaactct taatagggcc ggacgtggtg gcttatgcct	6300
gtaatcctag cactctggga ggcgaggcca gaaggatcgc ttgagcccat gagcccagga	6360
gtttgagacc agcctggcca acatggcaaa actccacctc taaaaaaat acaaaaatat	6420
tagccaggcg tgatggcaca cactgtagt cccagctact tgggaagctg aggagcgatg	6480
attacctgag cccagggata tcaaggctgt agtgagctgt gatcatgcca ctgtactcca	6540
tccagctggg ggacagagt aaacccctgt ctcaaaacaa aacaaatgaa aaaaaaac	6600
cttaataatc agtaactgtc actttatatt atgttgtgag tgtgtgtcta tatacaccta	6660
tatgtatata ttctcttat tacacattca ttggtgatct gatgtggagc cccagggtatt	6720
aagggaact ttgaactacc ctgacacaat caagccaaat atcattcccg tggagggaagt	6780
agagtatcta ggttctgtct cctagtgtga gctttacctt gaggacagag actctaattc	6840
agctgtgctg aaggagcaca tctcctgact tctgagcttt cccctggtaa attcaaatg	6900
gatgtcacgg cgccctcaga tagagcctgg taatttggcc tggggagagt gactgtcttt	6960
tggatctaatt ttgacttttg cccagttgg aggaaaatct tcagggttag gaaggattgt	7020
atttgtctga cccagagat aacctgggtt ttgaggaaca tggggcatca acctgaatgg	7080
tcttgtaaga tctctccac gccagcttgc cagtgtttct ctgatgaatt tagagtacct	7140
gagtagtgca ggcctgctgg gaggaggact ctccctctgt gctactcaga gaaattcatt	7200
cttcaaggcc cccttcacgc cttgctctta cccagctggg ctacagtta aataaaggaa	7260
atgacttttc ttctccctt cccccagtac ctttgttttc ctagtacag ggtgggctg	7320
gatattgaat ggagaaattg ctgggttcca tctaaactc ctccctcat ctctccctta	7380
cattacccea ttctctgtc tgcagccaca tccataatcc tgctctgtt agccttcga	7440
cagaccctca ggtgccagg acaacaggaa gctacttaaa gctggaacct cagactgtgc	7500
aatggaggcc agtgacaaa ctgaaagtag ctctgtcagt aattgtgctg gtgcgattag	7560
gcagctggcc agaattcttt ggatctctg gacatatggc tgactagtcc tcccaagcct	7620
tcccaacagg cctctttttt ttctttttt tcttttttt tttttcttc tttctttctt	7680
tcttttttt ttttttttag gctagtgaag tgaaattgtg ggagtggaaa aggaacaaag	7740
aatcggttaa ctggtagtga tcaattactt gtaaacacta ttgtacttgg accagcccag	7800
taggcctttt ttaaaactct gagttaccto tctttcctt ccttgagcag tgcattaat	7860

-continued

tctgtatctg gggcaatcct tctgtatggt ctctggacct ggctctctct ccttaggaga	7920
ggccaggaga gtagccagag agcatgtcat ttgtagctga ggttaaagtg tggagctatc	7980
aatggtgacc tggcctcttg gcattgttagc aagccagagg accttgacaa cttttttgat	8040
gattgtccgt tcacctgat caaagggtgt tggcttagga ggagggaaga aaagctaccc	8100
ctattagtct tgatggcccc agcgtgggtc tctattgctt gacctgggtc ctagcagcat	8160
tatcagaagg aaaatccacc gctcttaagg ctccctggaa ctttcaggac ttcctttctc	8220
aggattgcaa acataagact atttgagctt tcacttttga aaagcggtta ctaataccta	8280
tactctggga aagggtcaat gcagatagaa gactgtggtc actgcacag gcaacagacc	8340
atttccgcta aatttagtga ctccaggaag gccagtgaag aaataacaca cgtagcaacc	8400
agagactgtg ttgtaatatg ttggctgaca gcagggtact ttctgtgatg ctgaaagcca	8460
cattcatttt ctctcccttc atccccatct aagcaagcct ggtagaatca taattacagt	8520
aataggtacc acttattgag tactctgtgc cagacaccct cctgagcata cgacatgcat	8580
agcacattta atccttaca tgacttaata aaatgtagta ctagtcttac ctacttcgag	8640
aatagggaat tggagggttac ttgtttaaag tcacagagct aataggtagc atagctgaga	8700
tttgaactca ggcattctta ctcccttgct gcaagagtct cttggcattc ttgaatgcaa	8760
gcataattct taacctcact gaggtcagct ttcctcttat ataatatggg gtaaaagacc	8820
ctcaccctgc ctgccacaca ctggtagtgt cagataacat tgaagggtgt tagtttaag	8880
gcttcagga ctctataatg tcaacaaaag tgctgttaac tttctctctg gtctcaggct	8940
cctgatgtag agtcagtga gcaaccctgc catctgctgt tatgctgttg atgttgctgc	9000
cacacttact aacctaaacc ttgattctg gctgtggcct tctccagaag gtgtttactc	9060
atttgtccag tttatctttt aggaacacgc cagcccgtag atcattaagg ctggctattg	9120
gacagggggc tggggcctgc ctgacagagg aaggaaaggc agacatctgg ttcttctct	9180
gcccatacaa gagactccag cctgaccaca gactggtact cctaggatgt agcagcagca	9240
tatgagcttg aatgtgcctt aatcctgctc tttactttga gaagagagaa ctaaggaccc	9300
acagatgttt cacagcttct ataggaggca gaggtagaaa aatggagaga gatgaggcca	9360
gagatagata actgatatta attaaacgtt gtattaagaa cctcacttag attatctgat	9420
tcaatcttca taataacct gcaaccccca ctttttttg agaacagggt cttgctctgt	9480
tgtccaggct acagtgcact ggtacaatca tagttcactg cagtgtcaac ctccctgagct	9540
caagcaatcc tcccacctca gccttgcaag cagcttgac tacaggcgtg ccaccacacc	9600
ttgccatttt tttttatttt aagtagaaac aaggctctat taatactatg ttgccaggc	9660
tggctctgaa ctccagcgtat cctcctgccc cagcctccca aagtgtctgg gattacggaa	9720
gtaagccact gtgcctggcc agtgcaaccc ccattttata ctaaaacagg aaggcccaga	9780
aaggtttggg gtaacttgtc cagggtcaca cagatgatat ttgaactcag gtctccctgg	9840
ctcccaagag agtctgcttt ccactaggac tcccaggaga aaaaaaaaaa aaaaaacagt	9900
agacttggag acagaaaatc tgatttgagt cttagttgag ctaggctaac tgtgtaactg	9960
tgggcaagtt ccttagcccc tgtgagctc agtttcttat ctgtaaaatg tcataaaaga	10020
aatccatctc atggagtagt tgtgatgac aaggactctg aaacattag aatggtttaa	10080
tgtgaagat tagcagcagc acatggcaac attgtgcac ttatattaac tatccaaata	10140
tatcaagcgt catttgctat atataaaagt catcaaatca ggcactgtgg gggatacgga	10200

-continued

gttggcatac tagcctggcc tcttaattaa ttcattaatt agcttattta tttttgagat 10260
 aggtcttgct ctattgccca ggctggagtg cagtggcatg atgatatgctt actatagcct 10320
 caatctccca ggcttaaaaca atcctcctga gtagctggga ctacaggcac acactaccat 10380
 gccagctaa ttttttttta attttttgta gagacagggt cttgctctgt tgcccaggct 10440
 ggtctcaaac tctctgggctc gagatcctcc cacctgggccc tcacaaagtg ttgggattac 10500
 aggtatgagc cagggcacct ggctgggtct cttaactggt tccctaagac agctggaaat 10560
 agagaatgtc atggagcatt cctaaccatg ggctccagcc tggctttcat tctgtttctc 10620
 cctgaaaca acattccttt agtaatatc cgaataacag cttcatcagt ctgtctaccg 10680
 accactcttc aggtctcctc ttatatgacc tcccaactg cactaagggt tgtattagag 10740
 aaaagtggat aaagtccga gtcaggctgc ttgagcttaa atgccagctt cacttaccag 10800
 ccacctgacc atgagtcagc tgcttaacca ttctttgcca cagtttcctt gtctatgaaa 10860
 agggaaatgg ctccccacct aaaaagtgt taacattaaa ttcaatcatg tattcaaagt 10920
 cctgagcaga atgtctggcc atgactggga cttaacagat gtagcattt attattagta 10980
 tctgtcagtc ttgaaatggt ctctccctt ggctttcatg acattccaca ctctcctggt 11040
 tttctcttac ctctctggta atacctgttt gcttatcctt cttgtccag ctctgggatg 11100
 ttaccattcc ttcaggcgtg ctgttttctc cttaggcagt cttacacaca ctcatgactt 11160
 ccttcattg tctccacac actgatgacc ctaaaatcag tatctccagc ctaaaacctt 11220
 ccactgagtt ctgacctcat atgttgtact atcaacctgg cttgtccatt tgaatgtctt 11280
 ccaggcactt cagactctct tctctagact ttgctggact ttcactcttc cccctaaaac 11340
 tggctcctct tccactgaaa catgtatgct attgagaggc accaccatcc acccagtgcc 11400
 taagccagaa acctaggaat ccttgatacc tgttctctct catcctgcat atccaagcct 11460
 atcagtttta tctctaaatt atattttggt aggtttactt ctttcctttt ctccccacc 11520
 caccctgctc caagctacca tcatctcacc tggatgtctg caatagcctc atctcccaca 11580
 gccactctgc accccctaatt ctgttctcta tagagcagtt ggaaggagtg atttttgttg 11640
 tttgttttgt tttgttttag acagagtctc actctgttcc ccaaggctgg agtgagtggtg 11700
 cacaatttcg gctcactgca actctgcct cccgggttta agcaattctc ctgectcagc 11760
 ctccaagta gctgggatta aggcaccggc ccccatacc agctaatttt tatattttta 11820
 gtagagatgg ggttttgcca tgttgccaa gctagtctcg aactcctgac ctcaagtgat 11880
 ccacctgcct eggcctccca aagtgcgtgg attacagggtg tgagccactg cacctggctg 11940
 gaaggagtga tcttaaaaaa aaaaaaaaca aaaaaaact tgactgtgtc actctgtgtt 12000
 gtctctccta cctgtatac ttccacaact tccagtggt cttggataaa gaccaaatac 12060
 cttaacttgg ccaggcgctg tggctcacac ctatcatctc agcactttgg gaggccgagg 12120
 caggcagatc atgaagtcaa gagattgaga ccatcctggc caacatggtg aaaccccatc 12180
 tctactaaaa atacaaaaat tagctggtcg tggggcgctg tgctgtagt cccagctact 12240
 tgggaggctg aggcaggaga atcacttgaa cctgggaggc agagggttga gtgagcccag 12300
 atcacgccac tgcactccag cctggtgaca gagtaagact ccatctcaaa aaaaaaaaaa 12360
 aaaaaaaaaa ttccttaatt tggcctacag tagagccctc cgtaatgtgg cctctctcca 12420
 catctccaca acctcctgct cctgcactt cagcctcacc tctctcttg acaggccctc 12480
 cttctgacaa gggctttgtt cattctgctc cctctgccta gaatgcccc ttactctgtt 12540
 cacttaactc ctgcttatcg tttagatctt tacctggatg gctcagagaa atatagaagt 12600

-continued

aattcctcac cctgaaaaat aggttaggtc cctgttttat gttttcatag acctttcctt 12660
tgaggctttt tttaaaaaag tagttttaat ctcacattta ttcattgat catctcctta 12720
atgatattctt aagacctcta atagaacaat ttggtcatgg actgtggggg ttttgcccct 12780
cattgtgtca gcaactgagca tattgttggc ataggaggga tattgttga atgaattgct 12840
agagggtggc aagagatatg atgtaagtca ggcttttccc tgccttccc cttccccttc 12900
cccacatcct tcctatagca gccaccgtgg ctgcagttac tgtaaatggc aagacggaat 12960
cagttccgga cattgggttg ttttagaaaa ttgcctgcaa gtgtcagggt gataagttaa 13020
agctttgtct tttgcccctc gaggagctat cccatagtga gtagaagcca gagaagctga 13080
ccccaggagt ccttctttcc agcagcaggt cttgagctgc acttctctgt agctacaatc 13140
caggcaggaa caagccctag gtacctccgg agaggagggc aagagaggaa gaatgagttc 13200
agctactcta gccaccaaac tgattatgaa ttgccctgaa atctgaaaaa ttccaattcc 13260
aatcgtaagt ttgttttgtt tcattttgtt ttcttaaat gtatatttga aagatggcat 13320
taactaaaga tatatttca atatagagt gaaaaaatgg aatacttgca tagtatcttt 13380
tacttatagg tgattatga tggggagtgg ggtggatagg ttggcagttc ccccaagaag 13440
ttggaatga agttgtcct ctgtgagttg aactaattag atccacaagt aatgaaagca 13500
gtattgtgtt gtagttaaga gcacactcta gaaccagatt gcttagtttc aaatcctggt 13560
tctgcctttt attatctgtg tactttgggc aagttacttg cctttgtgt gcttcatttt 13620
tctcatctag aaaaatggaga ggccaggcgt agtggctcat gcctataatc ccagcacttt 13680
gggaggccga ggccggcaga tcacctgagg tgagaagttc aagaccagcc tggccaacat 13740
ggtgaaaccc tgtctctaca aaatacaaaa aattagccag gcatgatggc ggtgctctgt 13800
aatcccagct acccaggagc ctgaggcggg agaaacactt gaacctggaa ggcagaggtt 13860
gtagtgagcc aggattgcac cactgcactc cagcctgggt gacaagagct agactcagtc 13920
taaaaaaaaa aaaaaaaaaa aaactggaga tacaggctgg gtgcagggct tacacttata 13980
atatcagcac ttggggaggc ctaggcggga ggattgcttg aactcaggag ttccaagatc 14040
agtctgggta acagagcaag acctcatccc ccaaaaaaat caaaaattta gccaggcatg 14100
gtggctcatg cctgtggtcc cagctactca ggaggctgag gcgagaggat tgcttgagcc 14160
caggagggtt aggtgcaggt gaacctgac tgcaaccacta catgccagcc tggatgacag 14220
agcaagaccc tatctcaaaa aaaaaaaaaa aaagaaacga gccaggcgcg tttgtcacg 14280
ccagtaatcc cagcactttg ggaggccaag gcagggtgat caettgaggt caggagatcg 14340
agactagcct ggccaacatg gtgaaccccc atctcaactg aaatacaaaa aattagccag 14400
gcatggtggc atgtcctgt agtcccagct actcacttgg aggtgaggc acgagaatcg 14460
cttgaaccca ggaggcggag gttgcagtgg gccaaatca tgtcactgca ctccagcctg 14520
ggagacagag cgagactctg tctcaataaa taataaaca taaaataaaa taaaataaaa 14580
taaaataaaa taaaaaata tggaggccag caggcacggt ggctcacgca tgtaatccca 14640
gcactttggg aggccgaggg gggcggtatc caaggtcagg agatcgagac catcctggct 14700
aacacagtga aaccgcgtct ctactaaaaa tacacaaaat tagccaggca tgggtggcag 14760
cacctgtagt ccctgctact caggaggctg aggcaggaga atggcgtgaa cccgggaggc 14820
ggagcttgca gtgagctgag atcgccacc tgcagtccag cctggcgac agagcaagac 14880
tctgtctcaa aaaaaaaaaa aaaaatggag gttggcgcg gtggctcgcg cctgtaatcc 14940

-continued

cagcactttg	ggaggctcag	gcgggcggat	cacctgaggt	caggagtcc	agaccagcct	15000
ggccaacatg	gtgaaacctt	gtctctacta	aaattacaaa	aattagccag	gcacgatggc	15060
aggcacctgt	aatcccagct	acttaggaga	ctaaggcagg	agaatagctt	gaacctggga	15120
gatggagggt	gcagtgtgct	gagatcgccg	cactgccctc	cagtagagtg	agattccgtc	15180
tcaaaaaaaaa	aaaaaaagaa	gaaatggaga	tacaaactta	ctacctacct	ccttacaaac	15240
taccctcaca	gtattactgt	gaataaaagt	gtgtgtagca	ctgggaacac	tattcacaga	15300
gcactcatga	atgtttgttc	tttgttatta	gttactagag	aggcaaatgt	ctgccagggc	15360
tgaataatat	gtgtgaattg	gtgattgtcg	cacatatcta	aagaagtagt	tatttttttc	15420
aattaaaact	tagtttaaaa	accaatataa	ggccgagcgc	agtggtcac	acctgtaatc	15480
ccagcacttt	gggaggccga	ggtgggcaga	tcatttgagg	tcaggagtgc	gagactagcc	15540
tggccaacat	ggtgaaaccc	tgtctctgct	aaaaaaaaaa	aaaagtaca	aaaattagcc	15600
aggcatgatg	gcaggtcctt	gtaatcccag	ctacttggga	ggccgaggca	ggagaattgc	15660
ttgaaccag	gaggtggagg	ttgtagttag	ccgagtttgt	gccactgcac	ttcagcctgg	15720
gtgacagagg	gagacactgt	ctcaaaaaaa	aaaaaaaaaa	acaaaaacca	atataataaa	15780
taagtggcca	gcaatgaaac	agaaagtga	aagttagtga	agcaaaacta	gtactgtatt	15840
cagataaaga	tgctgaatct	agatttggtc	accagaatag	ggtcctttgt	ggcaacctgg	15900
gctagtttgg	ctgactcacc	actgccagga	tgaaatttct	ttcagtggct	actcatttcc	15960
ctttatttta	agtccatgct	cacagagcaa	ccttctgatg	cctaattcag	cttccctggga	16020
tacttaataa	caggaagggt	ctggaagtag	tacctgtata	ggggatatga	gtgttctgat	16080
tttaatagtc	aattcataag	tgtacagagg	gtttgatata	tggttaggtc	agaacctca	16140
cagaatgtct	acacctcttt	ggacattagg	aaggtcaaaa	acctgaaang	ccaaaagcta	16200
ggcctaagatt	agggtcattc	accaagaaaa	catcagcctt	gaagagttct	ctgggtgggc	16260
caccagtcaa	ccttcctttg	atcacacctc	cttcctcggt	gcttctttaa	gcattgacct	16320
gtaatgggta	tggaattttt	tgctcaccta	actccttcct	tttacagagg	aagaagttga	16380
agcccagaga	gatttaatgy	cttgccctaa	atcacacgca	gattttctgt	taaccagggt	16440
gatttttcag	gtgttccctg	ccagacgagg	gcttttttcc	ttgaattgcc	tagagatttc	16500
ttgagatata	cgaagcattt	ttcccagtgc	agcctggaga	aggatgtccc	tgtcaacaca	16560
gcatttggtta	ctcaatgtta	gacattcaat	tttctaatta	gtatcatgga	gcaacagtgg	16620
atgattatct	ataaggggtt	gcaattccat	gcttatgtgc	ttacagccca	tatagacaaa	16680
tatcagctgt	taaaatgaca	aggcagtaga	gatgtggccc	caggacaaag	gcatactctg	16740
ctgttagtga	acactagtgt	gccagcaaat	ttcacatggg	catatacacg	gccaactgta	16800
gaacttaggc	atttataacc	attcagagag	ccaaactggc	aactaaagat	cagcattctc	16860
tttggcattt	cagctttgct	ttctgttaaa	aatcactgct	tgcttaata	cctctgatag	16920
ctcttcactg	cctgtaggca	actcttttag	ctagcagact	tggtctttag	tgctctgccc	16980
ctactctctt	ccaccattct	ggcctcctgt	ctaattgctg	cccatagtg	ccatgcacta	17040
gagcttacag	acctgctcag	cggttatatg	gcataccata	ctctttatgc	ctcagtgcac	17100
ttgcacatgt	tggtccctca	ggccagaatg	cctgttactg	cctggcaatc	agcctattag	17160
agtctgcaa	taccatccca	tcttctgtgg	aggagcccc	cgccaaatcc	acccatacct	17220
ctccccacca	atcagagact	tcttctctct	ttgttatctt	cttcgttatt	ctcttcatac	17280
ctcagttata	tccatttcag	tattgtttta	cacatctagc	atcactotta	gagtgtgaaa	17340

-continued

ttctccaagt gtggagccgt atctagtttg tctttgtatc ccagagctta gcaaaagtgcc 17400
 tagaatgtag tgggtgctca gagtgtttgc tgggtgaatg atgtatttgt tgaacgactc 17460
 tttggacact tgaataaagt ccatccagta tgcaccatta ccatctcttc gctctacaat 17520
 attcttttag gcaagagctt atcttttgag gtgataagat aagctcaaac ttatgtagac 17580
 taagacctca gtctgtaaat gtcateccta agtcttaaac catcaaaacc agggcctcaa 17640
 ggaatggcat gccttctgca actgtagcaa cctgctgtgc ttattttgcc gtgtttttca 17700
 tttttcccc aaaagctaga gtcccttctc ccatgggcag tgctggaagt gtgctaacia 17760
 attctttctc cactactgctt acgattacaa aaaaaaccct cagcatctca tgccagactt 17820
 gagttaaggt tgttttcttt tgtgtgtcag ctgtattctg gtcagactt cctgatgatg 17880
 ccctatagag attttgtga gatcagaggg tgctccactg ccacagtag cactgactct 17940
 tgcagaagca ccgtttctga agttggctaa tgcacccct caggtttgtt tgtttgaaat 18000
 ttgttttagt tccagagata gcactttcat ggaatgacgc tatcttctag aatcactttt 18060
 ttttttttt tgagtggag tctcgtctg tgcagggt ggagtgcagt ggcacaatct 18120
 cagctcactg caatctccac ctccgggtt caagtgttc cctgcctca gcctcccgag 18180
 gagctgttac tacaggcgca cccccact cctggctaatt ttatgtgtt ttagtagaga 18240
 cgggggttca ccgtgttggc caggatgtgc tcatctcct gactttgtga tctgcctgct 18300
 tcagcctccc aaagtgttg gattacaggt gtgagtcacc gcgcctggcc tagaatcacc 18360
 tttttatacc ataactgtag caccactgcc gcgtcaccac ggaagagag aggcagctac 18420
 tgtggggtta caaatggga agagtggcac caggaaagtg aaagtctcta cttagccaag 18480
 gcttaacaaa atgtcaatca ccaaacattt atttattaag ctacgttcag gataagaaga 18540
 tgaacaagct atctgtacat tcattttctc gtttgaaca aggtaatgat agtgatctat 18600
 cctgcctgcc tctgagggtt attgtgagaa taaaatgaaa tcaagtggaa aagcacttag 18660
 gaaaaagaaa agcattggtt ttcaattgtt agtgtggatc agaaacactg gggcttgtt 18720
 aaaatgcaga ttcttagccc cagtctcagc gattctgatt ctgtatatct gaagtgggac 18780
 tcaggaaatct tgattttcaa caagctgacc agaggggtcca atgctgctat tccttagtt 18840
 acactttcag aaatattact gtaaatcaaa tggcaagaat aaatagtta tttgaggcag 18900
 ttttagtatg ttggacctg agtccaaaga cttgggtcaa actccagctt tgcagttcc 18960
 tagacctgtg acctaaaca gcaaccttct ctgtgaacct tagtccctc aggaacggct 19020
 ctggtcacct cctgctgtac tccattgatg actcaccaca taaggctccc tgggagtccc 19080
 ccaaaccttt gctctcttaa ctccctttac agcctctac atctcctgca ggtgctgtct 19140
 tctcctcctt ttccaggtc ctgctctgac acagcattca ttctcctctg ggaagggttc 19200
 ctcaaatgtg tctcaagca catcacccc aggaaggacc ctgtggccat atctgtctat 19260
 caccagatca aactactga aggcaggcac taggtactgt cagtggccag cataggcctg 19320
 gcccatacca ggtgtccaca gatgcotagt aaagaaacct atgattcagg acccccatga 19380
 tgagcaacta tagcactaga acagtataa taactaatgt ttataatgca tcttcagttt 19440
 acagagggct tttgtactca tcatctagtt tagttctgc aacaacctct tgaggaaat 19500
 agcacaagca ggacaaggga agccagaga tgttaataa tttatccaag tttatgctgc 19560
 tgggaagggc agcactgaaa taaaagaaa agttttctga gctcaaatcc catgcccttt 19620
 cctcaatgtg agctctagca aggtattcag gaatcctgcc tctacagttc agagcctcaa 19680

-continued

attgctgggt atgttgagtt ctgtatctg atttttctag atttctgccc cacattctta	19740
ctgtctggat atcaggaaag agtttatcaa atgcctgtgg aaatccaaga taaggctctca	19800
tgatgagtaa ccagtgaaa acatgaagtc aagtctaact agtcactact atttcaactac	19860
tgctgactcc tgatgacag ctccctttct aagtgtctac tgccactta ttecatcatc	19920
tgccatagaat ttatgtgaag gaatcaaagc aaaaggatca taaggcttcc tttttccagt	19980
atgtttttcc tcctttttga aaactgggcc agttagctat ctccattttt atttcatgaa	20040
tacatcccca gcgcctggta tatagtagat atggaacatt acactttgga gatattgcac	20100
ccattctcca gtttctcaa agttactaac aatggttcca tcaactgtcc aacatatttt	20160
cttttttcaa tatattggga aataattctc ccagctctgaa aatctgaaca catttcatgt	20220
gacttggtat cctcatatgt ctgggcttc caattctcca ttcctagttt caagtccatg	20280
aactgtaaaa caaaggatta gactaaatct ctaaaagttct atccagatgc caaattcttt	20340
tctctttcca tgatacctaa gatagatgcc aaatattgtc tttacctgg tgtttgtgaa	20400
catgacatca cttacagga gtagcagata ctaaactctc actctgtaaa aactgactg	20460
agttccatga gccagatact gaagtgaact gtctcacata tgttctcatt taatgctcat	20520
aacctgtga agctgggaat tgctgggaca ttttatttat ttatttattg agacggagtc	20580
tggtctctgc acctaggctg gtgtgcaatg gcatgatctt ggctcaccgc aacctccgcc	20640
tcccgggttc aagcgattct ctgctccag cctccgcagt agctgggatt acggggcaca	20700
caccaccaca tccagctaatt tttgtatttt tagcagagat ggagtttctc catgttggcc	20760
aggttggtca cgaacacttg acctcaagtg atctgctgc ctccagctcc caaagtctg	20820
ggattacagg catgagccac catgctgcc cgggaccctt gttttagaag gatgactgct	20880
gctataatgt agaaagtgt ttggaagagg ggaggagtgg ggcacgaaag atggttagta	20940
gatgggggtg gtaatgctta cctttcagta tttggaggct tcggagtcct caaaaattct	21000
cttcttgat tggagtctc ccagccaata gagggcttca cacaacagt tcttgggtt	21060
ttgaattggt tgaccagagc tttcttcga caaaagggtg gggtgattca ttcacttacc	21120
acaccttgcc tgaacattca ctggggctg ccggttatga aggtattgt tctccagcct	21180
gtcacagacg ctttgaagac ctgtgctca gctggttcta aggagtcagt ttgttcagct	21240
ccgtgccagg tttccaactt atgaatgtg ctggagatta acacctctcc tgccatttta	21300
tccctactat aattgccagt caaaggattc ctgcagttgc ctctggcagc cataactgat	21360
gaatgttctg ccagctgctc tgaggacctc gaagagcagt tttctatcca ggaccagttt	21420
ccaaggggtg gaggggtgaaa tatatcctcc agtgtgacat ttcctctccc agtgatgggt	21480
ggcttgggcc ctttgaagtt ggtctgagg aaccacacac ttgggtctga gcagccagca	21540
gcttatcaca tctggtgac aatccttcaa aggttcctcc tgaagtctga atttttgag	21600
gtcaaatgga ttccacctg gaggggcttc tgcttcaact caggacatgg ggagaaggct	21660
gttctcttcc cagggggagg cagttttcat ggcattgaga tgcctctca cttattcccc	21720
acccaccac caagtccttt gtaagaggag tagggggaga ggagagcgc tcagcctcc	21780
tgctcacatt cctagacacc gactcactga gccgctgcc gctggaacag cagagctgtg	21840
tgaatgtca agaggagtt tgcctatagg ctccctggcc tcagtctctt tgggtctgc	21900
atattcttcc attagtactg tgttcacac atggaatca gaggttaca ttaaaagata	21960
atttgctagt ccagactta atttggggcc cccttcttgc ctgattgaat tacaggggaa	22020
cataatagat ttttggtag aaatagttgt ctgtgtggct gggagaaaga ttgctcccag	22080

-continued

ctctccagct gggcagccct ttcagtatcc cgtatgttat ttccccactt ccagcccacc 22140
 tcacctctctc tgtggccctt gtgtgtcccc tcggctagga tcctgacctc ctgctcaaga 22200
 gtttaaaactc aacttgagac ccaaggaaaa tagagagccc tctgcaacct cataggggtg 22260
 aaaaatgttg atgctgggag ctatttagag acctaaccac gggccagaca gagagagtga 22320
 cttgtctaaag gccacatagc tagccacacg tagttgtaac aatagtctta atgatattaa 22380
 tggctaacat ttatcaacct ttaatgtgtc ccagactttg tgccaagggc ttacatgcag 22440
 tgcatgtctg cattcaaac cagacagtct ggctctgggc ccaggctgag ctttgggtata 22500
 gcatggtaga acgttgtcta taatgtctag tctgggttca aatcctggct tcaacttctca 22560
 catttacagc tgagtgcact caggcaagtg atttaacctc cctgtacctc agttgcttta 22620
 tctgtaaaga gaaaatcac agcactgtgg aatagtgggg gttaaaattc attcatacaa 22680
 gtagtgctgc aagcaatggt taatacaggg tagcacctg ttcagtgtct ccttctctctg 22740
 gctgcctctg gggctagagt gtggtgtctt cgtggtatag atagatagat atggctgagc 22800
 tctgcacaaa caccagagc tgctcttcac tattagaggt agtaaacaga gtggttgagc 22860
 tctgtgggtc tagaacagag gccggcaagc tatggcccat tgcctatatt aatacggcct 22920
 gtgattgatt gatTTTTTTT ttctttttga gacagagttt cactcttggt gccaggctg 22980
 gaatgcaatg gcacgaactc agctcacccg aacctctgcc tcttgggttc aagcgattct 23040
 cctgtctcag cctctcgagt agctgggatt acaggcatgt gccaccacgc ctggctaatt 23100
 tttgtatttt tagtagagac agggtttctc catgttgggc aggctagtct cgaacttcca 23160
 acctcagggt atctgcccgc ctcagccttc caaagtgtct ggattacagg cgtgagccac 23220
 catgactggc ctgattgact gattttttta gtagagatag ggtcttggtt tgttaccag 23280
 gctggtctca aacttctggc ttcaagcagt cctccctcct tggcctctcg aatgctggga 23340
 ttataggcat gagccactat gcctggccta tatgacctgt gatttttaaat ggttagggga 23400
 aaaaagcaa aagaatgctt tgtgacatgt ggaaattaca tgaactcaa atatcagtgt 23460
 cccagcctgt gcaacaaagt gagacctgt ctctacaaaa aataaaaaaa aataagccag 23520
 ggccgggggc agtggctcac acctataatc tcagcacttt gggaggccga ggcaagtgga 23580
 tcacctgagg tcaggagttc aagaccagcc tgaccaatat ggtgaaacco tgtctgtact 23640
 aaaaacacaa aaattagccg agcatggtgg catgcccctg tagtcccagc tacttgggag 23700
 gctgagacaa gagaattgct tgaacctggg aggcggagggt tgcagtgagc caagatcgcg 23760
 acactacact gcagcctggg caacagagcg agactccgac acacgcacgc acgcacacac 23820
 acacacacac acacacacac acgctgggta tgggtggccag cacgtgtggt ccagggatgc 23880
 actggaggct taggtaggag gateacttga gcttaggtgg ttgagactac aatgaacctat 23940
 gtttatacca ctgcacttta gccagggcaa cagtgtgaga ctgaatctca aaagaaaaaa 24000
 aaaaaaaaga aaaaatctt tccataagta aatatctgtt ggaacatagc catgtccctt 24060
 agtttatgtt ttatatatgg ctgcttttgc cctataatga cacaattgag tggccacgac 24120
 agtctgtatg gcctgcagag cctaagatat ttgctctctg gccctttaca gaaaaagtgc 24180
 cttgacctgt gctctagagc catatgtacc aggtttgaaa ctcagcctca cagctgggtg 24240
 tgatggcacg catctgtagt cccagctact ctggaggctg aggtgagagg atcacttgag 24300
 tccagaaggt cgaggccaag attgtagtga gccatgatgg catcacgcga ctccagcctg 24360
 agtgacagag agagaccctg actcaaaaaa aaaaaacaa aaaaaaaaaa caccctcacc 24420

-continued

acttatcagc tatttgcctt gagaatagtg acataacccc tcagaaccta tttcctaate	24480
tgtaaataga ggctgatgac gtttcctcct tttactggca atttaaacat gatggataat	24540
aaatgctaag cacttaacac agggcctaga agatattaac tgctcaataa atggtagctt	24600
cttaacagta ttcaaaccca tggctcttta tcacatgcat tgggtccctt gtgtccagtt	24660
ggtggaatgg gaaaaggctc ccttgtaacc ccatctacca tctttatcag actttcctgc	24720
catggttcac agtaagagat agaagctgca cggtgacttc tggctcttta caatggtgag	24780
cgggtgtgac ctggttaagg agagctgatg tcaactgccc aaatccagta gtgagatctg	24840
agtgttctgg tttcctccag cagccttgct tttccttta caatcctgca ggcagggaga	24900
caagggtttt ctacatgcta ggctctggtt tggctcatgt cacaactggg ggctgttcag	24960
gtgggctccc attccagata cctaggctta tcaatccctt ttggcacccc aggccttttt	25020
ctccctcatg ccccatTTTT cagtttgaaa agcatggtta tcacaggaca agtagaagaa	25080
gctccactgt ccaactgagg caatggatgg tgttctgcat gtgaacactc agtgaatagt	25140
gagtgaatga gagtaacctg ggctccatcc tatttgaga gagctttgga aaagattttt	25200
ctccttaaag agccagaatg aagcctggta gtgggagagc tccagctcta gattcacatg	25260
agcctacatt taaattccag ccctgccact gactcccttt ttgacctga gtgagttacc	25320
taatctctct gtacctcact tttcttgtct gttagagtggt aataattcct gtctcagaga	25380
aataaaagag tgcataatgt gtttgcaca tggagacaca tcagggtgag gtaatactc	25440
tgggccttgt ttccttattt gcaacacagc cctgccctgg agtgggaagt gcacctcca	25500
ttggtcagct cttgagctg tccccaggac aggcagaggg agggaaatga tgggagccct	25560
agtgccagga cagaacagat ggcagctcag agctaggatg gctctctgga cctgtctctc	25620
ctaccagagg tcccccgctc tgggtgggct cttcctggac ctggcatcct ctgctttttt	25680
tttttttcca cctccaagca gaattactgt cctgtaggca gctcctctgc ttgaggacat	25740
ctggggccag atatgttacc actctatcct gccttgccct tccctgagct caggatggac	25800
gctcaattgg tcccagttat tgtctgcagc gcctgcctgc agcctcgatc cagcccagct	25860
ccacccttg cctgcaaggt ctgtttccta acagctgctc caaccacaca cctcggttct	25920
gcgaggagcc ctctctctcc tccctccctc cctcattcag ggggtgggact gaagaagaag	25980
gctaacttga cagcagcgtc tctttcttag ctatgcaccg gccctgctc aagaatgcca	26040
gtgtgtgtgt agcctccaca gagaggtcgt tttctcggag tccagagggg ccgcctgagc	26100
ttctgagaac tagggaggag ccatccagc catgagcccc tgggggaatc tgctgggggc	26160
caagtggcct ggagtctca ggctcccgca gctgctccgg agggagaggt gagctcaggg	26220
cagcctgcct gcagccagag gtgcccggag ccccgggcct gtcattggtg ccatctacag	26280
ccggcctgag gcagtcacag acggatttgc agctgagcct gtctatctgg tgggggaaga	26340
agatggggag ttacttgcga gtcccggctt acttcacctc cagagacctg tttcgggtgag	26400
ttggtctccg agttccctc tccatctctc ctggcccctg gtccctgagag gaggggtggtc	26460
tccctaaatc tccctctcac ttatgccttt accatcggtt ctgcccggca gaagccagcg	26520
gagggttata ccaaggagaa tcggccttgt gaggtacccc cattatgtcc tggaaagtgt	26580
gaggggaggg atataccag aaggaaactc ttagggagct ccagctcccc ttctatccca	26640
gacaaacctg aaggagcctc caaaagatgc cactgacctg cccattgtag atgttactgc	26700
ttccgggggg aatagcccaa atagagtgtt gtttccagct ctacatgtc ttacctgagg	26760
gccatgctgc ctgcccagga atttgtccca acaagcagga tgggcagggt ttgccaaact	26820

-continued

gtggaaactg gcaagtcctg ggtgtgggta gcctgtgaca cagtaggcac cttataaacg 26880
 tttgttctct taatggcagg cacatttgcc tctggccttg aagggcttct gagctcccag 26940
 gtgaatgtag ttgctgggga aagacctggg cgagtgcctc taagactgga gcaatgggct 27000
 ttagagtgtt cctgagctgc tgggccagcc cccacacctc ctcagtcctt aggcctaagt 27060
 acctccacga gcctctctct gtggggcttc tcagagggag atgtggaaac tctacctcta 27120
 acctggcttt ctttgctcat tgcccactc cactcccat agaaactccc caggggggtt 27180
 ctggccctct gggtcccttc tgaatggagc cattccaggc tagggtgggg tttgttttca 27240
 ttctttggga gcagcctgtt gttccaaaaa ggctgcctcc cctcaccag tggctctggt 27300
 cgacttttcc cttctggctt ctctaagcta ggtccagtgc ccagatcttg ctgccgggat 27360
 actagtccag tggccaggcc ctgggcagaa aagcagtgtt ccatgtggtt ttgtggaatg 27420
 accggaccct gtagattgc tgggaagtgt ctggacaggg ggaaggggga agggaactgg 27480
 tcctcaatgc tgactctacc aagcgccctg ctagacactt tctctttaa tctctcaaca 27540
 gcctaaagag attatatac cccattttac agatgaggca accagtttca acagagttaa 27600
 catatggagc ctcactgggc agctttttct gtcttctga ctttctctca tcttcaggg 27660
 ggctgcagg tttgttttct ctcctagtgg agaggaaatt ctcaggtttg ttttctctc 27720
 ctacagagag gtaaaaaaag ggatagtttg cctgacttgt tgaagggttg gctgagattg 27780
 ttttctaaag agccaatgga aattgatctt gagtttagga gaaagctttt acatgtggaa 27840
 ttaagatgcc aagtgttgaa gttagccacat ttcaggtcct cattaatttc tcttaacct 27900
 gggaaaggcag cttaggagaa ggggtgttcc tttaggagcc aggaactata ccccttttac 27960
 ccttgagag gcaagggaagc caggaggagc acaacttctc aggaagagga gaagctagag 28020
 cagatagtga actctcaacc tgaaccttta agggccagac cactaatgcc acccaagtc 28080
 acctgccgtt tgtcttgttc gtccaggc tttctggaga acctgatctt cttgccctta 28140
 cccccaagct cgtttgtccc agctagagtc tggggggtag tgaactgact tcgtagacat 28200
 tcttcccttc cccaaataag aggccacatt cctgaagtca cttctgaaga gatagctgcc 28260
 acacagggct ctttccccc agggaggagc caccagacc cctgtctctc ccaggtatcc 28320
 gttaccacat cactacctg tcagaaagct gtttctgcca ttagccctc cctcttttat 28380
 tataggatat cctcaagggc tctcttttg gcctcagttt cctcctggc agaaagtaga 28440
 agctagactt cttgggtccc tgaacagggt ccttgctgga ttctgtgaa caaattaagt 28500
 tcttgacctt aggcctctgg gggagtacaa agtctatggg agttctgggg ctgtggttg 28560
 aaggaaagt acgcaaccag attccatggg gacatgatca ggcgtgacat gtgaggagg 28620
 aagagggagc aaggaatga agaatacaac ttctgtgtcc catacacccc tgctgacag 28680
 gccatacata ctcagcagag aatgcactgt ctttctacc acactagctt gaggagttag 28740
 ctgcaattac cactgtgctt ccaagtaaga aaatacctca aattggaatt tacaaaagag 28800
 gtaaatagg gagtggcttt gtccggacat ctttaagca tttttcttt tatagaattt 28860
 cacttaatgt ccaatactga ttaatgagc ttgggtttac acattatctc ttgaagaaaa 28920
 caaatgaacc tttgtgttcc aaagcaatcc atgtttaaag ggaaaaaatt atgcataact 28980
 ctgccagct tcacagtaac ctttggcagg tgccttaggt cctctgggac tcttttctt 29040
 atctgaaaaa tgaaggactt ggatcagggt aatgggtccc agctctgcaa cttatgtgac 29100
 tcctcagagg cacacaagct cttttccatt atttgccaaa taatggaggc cctgtcttta 29160

-continued

actgcagtac aactacacaa aatacttgaa actacagtct tcttggtttt tggttggaac	29220
tgaatcagtg cactctagca acacttattt cttgctgttc gtaggcttca ttatgtgttt	29280
ggtaattttt ttaaaacaac aataacatat tccataataa ttacagctta attggcagac	29340
tgtttcagtc tataggatct gcaggaagga ggagtaataa agggattttt gactgagctc	29400
ttatggaaca gagtctctct agggccctgt catatctgcc cttctgggcc ctggggaaaa	29460
gttggtatcc ccagttgttg tgctctccag gtgcctcag gctgtggtgg agggagcttc	29520
ccattctctc cttcagccca ctcaattcag aggctagggg ctgaaagaag cttctctaca	29580
actggctgtt cactgggagg ttaagggatg accatccagc caggccttcc tcaggacatg	29640
ggagggtcta tgccttaaca tgtgtaaatc cactgcaata atgactggtt cttttacccc	29700
ataaggttga gaatttacct gtaaacattt ttgtctgaag aatttggtat taagtgggg	29760
ctgggcctct atcttatctc acttggtctc tctcagcaca gcacctgccc tgcttgttct	29820
tacacatcct agatgcacag taactatttc ctaattatta gaaatctatt agaataaatt	29880
gatttcagct gggcttggtg gctccttctt gtaatcccag cactttggga ggctaaggct	29940
ggaggtatcc ctgagtcag gagtttaaga ccagcctggg caacataggg agacctgtc	30000
tctacaaaaa ataaaaaatt agccaggcat ggtggtgtgc acctgtagtc ccagctactc	30060
aggagggtga ggcaggagga tctcttgagc ctgggaggtc agactacagt gagcaatgat	30120
tgtgccactg cactccagcc tgggtgacag agtaagactc tgtctcttaa aaaaaaaaaa	30180
aaaaaagttg atttctattt ggatagataa ataattcatt ttaggacctt totttttcac	30240
ttacagaaat ctgtttcatt ctgggctgag aagcaggctc atattgctag gcataaggaga	30300
aaaaggggtc tgtctgcatt tgcccttggt ggtctcaaat tggggaggga aagaaatgaa	30360
cacttactgg ctacctcttg tgagccaggc atcatgcaag acatctgtac ataatttaat	30420
tctcataacc ccataagata ttattagcaa tgtacaagtg aggaaactga ggctcagagt	30480
catgaagtaa ctggccttgg gtgacacaga tggtaaatgg cagagaagga atatggatcc	30540
aggtcttgaa agagaaaatc tcaactgatt atctttttta aaaaactcat atgttctctg	30600
ctgactcaaa aggtctctgt gtggtatctg gttgacctac tgaactgacc atcagggttc	30660
catgcacttt gtatctgccc aagccctcag aaccctcag taatgttttg gaagatgagt	30720
tttgagggtt gtccttaggc atagcctcag cgtatgtagg cctctagggt atctccctta	30780
acctgaggat ttcagctcaa ttcactctgg ctectcagga cagtgggtat actggttcag	30840
acctcagctt taccacctcc cagctgggta ctcttctacc tacagccagg gcagattttg	30900
actttcactt gaaacttcca aaaattgaaa ggtagaaaaa cagccttgcc tttgggaaga	30960
acgtatgatg tccatggcct ctaagcatct gaggtgggac atgttcgagt agcaccttac	31020
agttccaaag tgtgttctgg gttctttgtt taaaagaaca gagactgctg gggaaattga	31080
cactgtgaag tatatgaag aggagaattg tgctatttaa cattcagtac ttgggctaaa	31140
ggagaagcat cacgaagtgt taactactaa agggcttga gctgtcaggg ctccagcttc	31200
cttattttca caggtagaaa tcttgaggct cagctgttga gatgtgctgt ctactccgg	31260
tgacatagta cagtggatgt ggctttgag ccaagcacac atagcttcac attccagctc	31320
catcaattat gtattgggca gctttgcaga atgatttgac tttaactctg cttttcagtc	31380
ttctgtaaaa cagggaatat cctgctaccg tagggtgtgc aggattagag ataataaaa	31440
taaggtaacct catataggac ctggattatg gctggcattc aataaatagt agctgttaat	31500
tgatagctaa gctagaactc tgaagtctac catggcaact tcttaagtgg tctgagaacc	31560

-continued

cagttgtgtt ctgtggcaaa acacagctta gggatccata cccagccctc ctgtcagctg 31620
 ttcaccttcc agttcttcag agacatgtgt ggcagtgact ttggccacat agctggctgt 31680
 gccctttaa ggcattcctt gacacagata tgtggactgg tgacgttgct ctccagccag 31740
 gtgttcttcc cagcaggctg gccctggctgt ctctgcatg cctgtacttg tttgtctccc 31800
 tgctccctct cctgggctg gccagagcta cttgcagcaa acaaaagcag gatattggca 31860
 atggaagga ggggtgttgc tgggtctccc atgccctgcg gcgcacatac cattgcaagg 31920
 gcgtaacaga gccagggcct gcatttgggt gcaataaagt ctgcacacag aagaaaagaa 31980
 ggacgtggg accagagacc atggaaccct tgtgctcccc tacctgggct actggttctt 32040
 gccactccta ccattttcag tttggaata tttgtaagg ctttgcctt ccaggctcct 32100
 tgcttgggc tgagtctacc aagagtaagt gggatgctgt ttttgcctc agggagctaa 32160
 cagtctagt aagaagaaag atggttgccc aggaacttct aagtcagaag gcaggaggca 32220
 agaaggaag ccctgctcct actgccagcc ctctgttggg caccctatag ttcttcagaa 32280
 ccacatttaa tcctcactgc aggccaggca tagtggtca cactgtaat cgcagcactt 32340
 cgggaggcca agcgggcag atcacttgag gtcgggagtt cgagaccagc ctcaccaaca 32400
 tggggaaacc ccgtctctac taaaaataga aaaattagcc ggggtgtgtg gcatgcgcca 32460
 gtaatccag ctactcagga ggctgagggt ggaaaatcac ttgaactcgg gaagcagagg 32520
 ttgcagtga cgagattgt gccactgcac tccagcctgg gcgataagag caaaattcca 32580
 tctcaaaaa aaaaagaaaa aagaaaaaat cctcactgct acctgaaag taggtgatga 32640
 cattgccatt tcacaaatga gaagtgaagg ggctagccca agatcactta ggtggtaaat 32700
 ggtggtgcta agattagaac ctcatatcat ctagggaana acacagatat gcacagagtt 32760
 aaggggaccc aggttattgt ttgtcctctt gtttcacagg tggggaaaca acccagagag 32820
 ggaaaggggc ttgtccaagg caatttagca cccaagaact tgaaccata tctctctcct 32880
 cctcatttag agctcatccc acatgtatct tatattgaga ggagtgtgag ccacatacca 32940
 agaacagtct tccccctgc ctccaacctc actgtgcagt tttgagacac ttcacagcca 33000
 tactcttcat gccataccca gcccttaaga ccctgaagt ccccttcoat aagacaagta 33060
 ggaaaagcta tagggtaaaa atagccatca gtgtttgtt agcaccagc aggaattggg 33120
 cactccagaa agataaagg attctcagg acttgcttct ctgacttcc ctgctcagc 33180
 tgcttcaact cattcctgcc cctctctct acctcccga gtgctcagaa gtatagaac 33240
 tcaactgtgc ctctcacctt gcattgttga gttttattha gactttctct tectcaactc 33300
 ttcataagct catgaaagg gaagtagggt gccctgtgta tttatcttt atctctgag 33360
 tgcttagcaa gttataataa tgcacttgcc tggcaaaagg cttctctca tacattagct 33420
 tatttctct tcacattgac tctttgtagt aataggatgc tattagttat tttcaatgag 33480
 agaaagctac taagagaagt tgtccagcta gtgacagtaa gtggctgata aagtgaagct 33540
 ccattacatt gtcacatct ttaatagaag ttaacacata ctgagtttct actatattg 33600
 gtctttttt ttttttttt tttttttta gagacggaat cttgctctgt tgtccaggct 33660
 ggaacgcagt ggtgcaatt tgggtcacca caacctccgc ttcccagggt caagcgattc 33720
 tctgcctca gccctctgag tagctgggac taccagtga cgccaccag cccggcta 33780
 tttgtattt ttagtagaga cagggttcca ccatgttggc caggctggtc ttgaactcct 33840
 gacctgtga tctgccgcc tcagcctccc aaagtgtgg gattacaggt gtgagccacc 33900

-continued

gcgcctgcc	tatattagga	cttttatata	agctatctct	agctagctag	ctagctagct	33960
ataatgtttt	ttgagacaga	gtctgactct	gtcaccacag	ctggagtga	gtggcgtgat	34020
ctcgactcac	tgcaacctcc	acctcctggg	ttcagtgat	tctcctgcct	cagcctccc	34080
agtagctggg	attatagggt	catgccacca	cgcccagcta	attttttgta	tttttagtag	34140
accaggtttc	accatgttgg	ccaggctggg	ctcgaactcc	tgacttcaag	tgatccaccc	34200
gcctcggcct	cccaaagtgc	tggtgattata	agcataagcc	actgtgccca	gctgctctct	34260
atatttttaa	tacataattt	ttccattaat	tttcacagca	gttcatttta	tagatgagga	34320
aaactaggcca	gagaagttaa	atatcttgcc	caagatgatg	taactagtaa	gtggcaggat	34380
caagattcaa	accaagcaat	gttcaaacct	cttggaagca	agaatgtggc	cactgtggaa	34440
ggtgcaaggc	cttgacaaca	agaataggga	aaagaaggaa	ctagaaggaa	agagatggca	34500
tggtgctcagc	aggccaggga	gctcttagct	gtgtgtgttg	ggaagctcag	aaggaggagaa	34560
gaggttgtct	gtgcaggtaa	gtcctgagaa	cacaccagac	ttttgagagg	tgagcttca	34620
tagccaggtc	attaggggag	aagggagcta	tagatttttt	tttttttttt	tttttttttt	34680
tttttttttag	agacggggtc	ttactatgtt	gccaggctg	gtcttgaact	cctgggctca	34740
agtgatcctc	ccacctcagc	ctcccaaagt	gctgggatta	gaggcatcag	ccaccccgcc	34800
cagcgagcta	tggtatcaac	atgtacatct	tacacagtgc	taatagaatg	ttgggtttct	34860
tccccaatat	tttattttga	aaaaaaatc	aaatatatag	aaagtgtgaa	aaatgtagtt	34920
caaagaacac	ctacataacct	ttcacataga	ttcatgattt	gttaagtta	tgccactttg	34980
tatatatctc	tctccctcct	atctgtatac	ttttatttat	ttatttttgc	tgaactattt	35040
cagagtaact	taaaggcctc	ttgattttac	ccttgaacag	ttcaatatgt	ttctgctaag	35100
aattctccta	tataagtcag	atatcattac	atctaagaaa	attcacggca	attttacaat	35160
ataatattat	agtcacaatc	catatttcct	cagttgttcc	aaaaaatgtt	catggctgtt	35220
tcctttttta	atctaattt	gaatccaagt	ttgaggcatt	gtatttggtt	gctgtgtctc	35280
taggggtttt	aaaatctgtg	cctttttctc	tcccatgac	tttttagaag	agtcaagacc	35340
ggttattctt	atagaataac	ccacattcta	gatttgctg	attagttttt	ttatacttaa	35400
cgtatttttg	gcaagaacat	tacattggta	acgtgttgg	tgatgggtca	gttttgaaga	35460
gtggagatga	ttaaactgct	ttgttcatt	gaagtatctg	tcaagaccag	agatccttaa	35520
ctggtgccat	aaatagggtt	cagagaatcc	tttatatata	cacctgtcc	cccacctaaa	35580
ttatatacac	atctctctta	tatatcatt	tttctagggg	aggcttcttg	gcttttatca	35640
aattctcaga	gggcccacag	acccaagag	gttatgaaac	actagtctgt	ccactgaggc	35700
aggcaacaca	gagctgggtt	ctggggcctt	gttcagctg	aaccagcttc	ccttggggag	35760
atagcacaag	gctgtaactt	tgccccatct	tggttttggg	tcaaagagga	ctgtccattt	35820
tggtgtcata	cctaggaacc	agggacagct	tatgtgcct	ggttcaggg	atccaggaga	35880
atctcagttc	ttgtcttgcc	tttcagggtg	tcagaatgcc	aggattccct	caccaactgg	35940
tactatgaga	aggatgggaa	gctctactgc	cccaaggact	actgggggaa	gtttggggag	36000
ttctgtcatg	ggtgtctcct	gctgatgaca	gggcctttta	tggtgagtga	atcccttcat	36060
atctgcccct	cttggctctc	agagtccatt	gacagtgcct	ccagttccct	gtggcctgtt	36120
aatcttttag	tctttccatc	agccagggca	tctcccttta	tttatcatt	cattcaacta	36180
gcaggtatca	attgagcacc	tactaagtga	aagtaagat	ccttccctca	aagacttaat	36240
agttgaacgt	tggtgagtgg	aggagaggca	ggcagagagg	agacacaata	tagttggata	36300

-continued

aggacctcca aggagagtgt tacaggctga gaggaggata tacttaggtt gtcttttaggg 36360
 aatcagaaaa ggagactctg gaataggctg gcagagagag gggctacctc ctataacctgc 36420
 tctggacaaa cgactttaag catagtgaac gatttgccaa ccctgtattg gaagaactga 36480
 tcttttttag tggggatgat tacttctggg gatttcttct cataactgag accaaaacag 36540
 ttttgtgcag tctcagaaat gacaggaggt accaatctga cacttccttt ggaagctcta 36600
 gggcagagag tgaagagtg gattttgacg ggggccttgc ttggagggtca ttcacccacc 36660
 cctgtcctca ctccagcaac agtgataact cacttccttc ctccctttgt acacccttct 36720
 cccacactgc tcacagggtg ctggggaggt caagtaccac ccagagtgtt ttgcctgtat 36780
 gagctgcaag gtgatcattg aggatgggga tgcataatga ctggtgcagc atgccaccct 36840
 ctactggtaa gatagtggtc ctttgtctat cctctcccat ataagagtgg ctggcgggga 36900
 gggacagtgg cagggtagt tgggcagaa gagtgttagg gtatgcagag cattggattc 36960
 ttaccacagc agtgcctta accagctctt taactgttaa gcagaatgat ttacacatgt 37020
 ctctaccctt tttccttacc aaccttgaaa atgtcttcac tctgcctgc aatcctccca 37080
 gtgggaggca ctctcaagg acgatccag aacattaaag tcaagaccc cttagagctc 37140
 accctgtcca accaccttg ttgataaag aagtcagcct ggggcccag gaatagaata 37200
 gtacaagggc aaggttctca ttgtgagtca aaggtagagt gaagagaacc cagaccatct 37260
 caccaccaac caggccagt tttttccaaa tataccactt gctgcagatc tagctcagca 37320
 cccacagtcc cagccacccc tgagaaccca ggctcctcat tctgagcagc cagctagaat 37380
 catgacaaag aggggtgtag tgagactatg ggtactgttg cttaagacca catggtgcag 37440
 tgggtgctgg ggggcttctg tgtgggactc tagcatctta tccccctg tgccctctcc 37500
 ccagtgggaa gtgccacaat gaggtgggtc tggcaccat gtttgagaga ctctccacag 37560
 agtctgttca ggagcagct ccctactctg tcacgtcat ctccatgccg gccaccactg 37620
 aaggcagcg gggcttctcc gtgtccgtgg agagtgcctg ctccaactac gccaccactg 37680
 tgcaagtga agagtaagta ttttgagaac ccttcagcag ggggtcttga gcagagtctg 37740
 taaatgggcc tcagagggct tagacctcca aagtctcatg cagaactccc ttattctca 37800
 tctcatatct tttctctgga cccactatg ctgtaaccgt acctgggctt tggcacttac 37860
 tgttctctct gcccaggcta ctctctacc gatacttaag gcaagaatca ctacacttcc 37920
 aggtgtcagg tttcaggcta tgtttgtct ttgaatcat ctgcttcat tatgtgtatt 37980
 agttgtttat ctctatccc ctccactaga atgtaaatc cagaagaac ttgtgtctt 38040
 attcagtgtc gcatgccag ggcttggag agtacctggc atatagtagg agttgattga 38100
 ttattatttt gtcagtcag agaatgaat gagaaaatgt ggtccatggc ccaaaagaag 38160
 ttaagacct atcctagatt caggccagag accagatgga gaaagagtct gtgtctatct 38220
 aataccagta atgtcgtacc tctggccgct taccatgtaa atattgattg tgtatctacc 38280
 atgtgttga cactaggcta gtgttgcac agcaggtgaa agatactaga gtttgggaag 38340
 tcaggaggag ctaaggctg ttctacaacc ttattagatg aagaggagag ggaattgtgt 38400
 tcagggcaga gggagaagca tttctccaaa agtaggagtc ttaatcatgt ctgatgtagg 38460
 ttgagtgtgg ccagaaaagg ggctgttaag tatagagggc ctggattatg aaaatccagc 38520
 agatccattg agagtttaag cagcaagggt ttgtgaccaa gttaacattt tagaaggatc 38580
 actggtatgg aggttgatt ggagagggga aagcctaaag gtatagagac tagttaggaa 38640

-continued

gctattgtag gctgggcatg gtggttcacg cctgtaactc cagcactttg ggaggctgag 38700
 gtggggaggat tgcttgaggc caggagttga agaccaacct ggccaacata gcaagacccc 38760
 gtctctgttt ttcttaatta aaagaaaagt ccagacgtag acatagtggc tcacgcctgt 38820
 aatgccagca ctttgggagg ccaagggtggg cagattgctt gaggtcaaga gtttgggatt 38880
 agggccaggcg cagtggctca cgctgtaat ccagcactt tgggaggccg aggtgggagg 38940
 atcacaaagt caggagatca agaccatcct ggctaacaca atgaacccc gtctctacta 39000
 aaagtacaaa aattagccgg gcatggtggc ggacgcctgt agtccagct actcgggagg 39060
 ctgaggcagg agaatggcgt gaacctagga ggcggagctt gctgtgagca gagatcacgc 39120
 cactgcactc cagcctgagc gacagagcga gactccatct caaaaaaaaa aaagagtttg 39180
 ggattagcct ggccaacatg gcaaaacccc atctctacaa aaagtacaaa aaaattagct 39240
 gggtaggtg gtgcgcgcct gtaatcccag ttactcagga ggctgaggca tgagaattgc 39300
 ttgagcctgg gaggtggagg ttgcagtga cccagatcat gccactgcac tccagcctgg 39360
 atgacagagt aagatgccat ctcaataaaa aattaaaaac aaagttaaa aaaaaaatag 39420
 aagctattac cgtgatccag gtaagagatg tgaataacta caatgatgga aagaaggcag 39480
 agttcttaga gatgggagta ggagagatga gggaaactcca gattgggaag atgatgtca 39540
 agtttctggc ttaggccaca gggtyagtgg caattccctt cactgagatg gggcatcctg 39600
 gaaaagggtg tgctttctg tgtgggtatc ctgggcccct taggggccac tggtgccctg 39660
 ggacctggta aaccttcct gcacaagcag aattggtcaa gcagggtttt aggacatctt 39720
 taccctgcct caactctgt ctggcccagg gtaaacggga tgcacatcag tcccaacaat 39780
 cgaacgccca tccaccctgg ggaccgcac ctggagatca atgggacccc cgtccgcaca 39840
 ctctgagtgg aggaggtaga gtgtgtgtct aatctgtctt gtgagggtgg gacatggaac 39900
 agatcctctg ggaaatcagg ctgtagcctt taccttttcc tacccccagc ccatctcttt 39960
 gtcttagcat tgagcctgtg accactgggtg acctatttca gcgtaacagg tcccagggt 40020
 agcagggatg gttgatggac gggagagctg acaggatgcc aggcagaggg cactgtgagg 40080
 ccactggcag ctaaggccca ccattagaca agttgagcac tggccacact gtgctgagt 40140
 catctgggtt ggccatgggt ggcctgggat ggggcagcct gtgggagctt tatactgtc 40200
 ttggccacag gtggaggatg caattagcca gacgagccag acacttcagc tgttgattga 40260
 acatgacccc gtctcccaac gcctggacca gctgcggctg gagggccggc tcgctcctca 40320
 catgcagaat gccggacacc cccacgcctt cagcaccctg gacaccaagg agaactctgga 40380
 ggggacactg aggagacgtt ccctaaggty ccacctccca ccctggctct gttctgtcct 40440
 atgtctgtct ctcggtgaa gctgagctgg ctttcagaag cctgcagagt taggaaagga 40500
 accagctggc cagggacaga ctatgaggat tgtgtgacc cagctgcccc tgtggggatc 40560
 acagtttaca gccagagcct gtgcggaccc agctgtctgc caggtttcct tagaaaacctg 40620
 agagtcagtc tctgtccact gaactcctaa gctggacagg aggcagtgat gctaaaccct 40680
 gaagggaac atggcctatg gagaagcat ggagctcaga gcctggagta cgggcacaga 40740
 taggattgaa taaattgtgt agaagactt tgaatacaat aaagcaaaag atgaatgaac 40800
 gtttttttta gacttgaggg accaacaacc cccaaacccc agattctgc aggtccatgg 40860
 ggaaggagaa gttgccttga gtggaagccc caagtaggga gacttacaga aaagaagtca 40920
 agagcactgg ctcccaggca gaaatactga taccctactg gggcttcagg ctgagctcct 40980
 cccttcacaa atcacttcat ctctctgagc ctgtttctgc atctgtgaca taagatggta 41040

-continued

agataaaggt ggctgtctca ccaattatgt aaggattaaa tgtggaaaag gacataaagt 41100
 tgtatagtgc tgccataggg acagtgttca gtaaacgtga cacattctta gtatcactaa 41160
 gaatcaggtt cttggccagg caccgtggct catgctgtga atcccaacac tctgggaggc 41220
 ctaggtcgga ggatggcttg aacacaggag tttgagacca gcctgagcaa catagtgaga 41280
 cactgtctct aaaaaaaaaa aataataata ataattgttt ttaattagat gggcagggca 41340
 ctgtggctca cacctgtaat cccagcactt tgggaggcca aggccggagg attgcttgag 41400
 gccaggagtt caggagcagc ctgggccaca ttctgtctc tacaagaat aaaaaagtta 41460
 actgggcatg gtggcacatg cctgtaatcc cagctactca agaggctgag gaggaggatt 41520
 gcctgagccc aggagttaa gactgcagt agccttgatc acaccaactgt actacagctt 41580
 gggcaacaga gtgagacctt gtctccaaaa aaaaaagttt gttttttttt atccactctc 41640
 ctcacaaaac aaactgagta agttagagcc ctctcagctg gcatgtgtg gaaacagtgc 41700
 cctctcatta aagtgtgcc ctcactocca ttgctcttg gccttggta gtatgatgaa 41760
 attagtggga ggcaggggcaa cagagggcag ggaagagcta gaaatccatg gcctggaaaa 41820
 gggaaagattt gggagtggcc aggtatctgt agagccacca tgcagaggag gggggcagct 41880
 agccttgtgt gctctggtg gcatgtgcag caggaggcag agcaaaagga caagggttaag 41940
 .taaacctgta ggtcgggaca agccaagagc catccagcgt cagtcctctc tgggtagccc 42000
 aagtaagca gtagcatacc ccagagagaa agttcgagg gctgttcacc tgcagtgtg 42060
 tggacttcaa cctctctgtt cctctctcag taagtgaata taacagtcac tgaccatgac 42120
 tattatcgac cgcttttgaa aatgtaaaac tagtgacttt attgctgtaa aaatcatagc 42180
 tgtttatcat cttaaaattc aggaacatg gacaggtaaa aagatgtgca aaatatcatc 42240
 caaaatccca tttgctggcc aggcacgggt gctcacgctt gtaatccag cacattggga 42300
 ggccgaggcg ggcaaatcac ttgaggtcag gagtgtgaga ccagcctggc caacatgggtg 42360
 aaaccctatc tctactaaaa atacaataat taggctgggc gcagtggctc acgcctataa 42420
 tcccagcact ttgggaggcc gaggtgggag aatcacaagg tcaggagttt gagactagcc 42480
 tggccaatat ggtgaatacc catctctact aaaaatacaa aaattagggc cgggtgtggt 42540
 ggctcacgcc tgtaatccca gcacttaggg aggccgagac agatggatcg cgagatcagg 42600
 agttcgagac caacctagcc aacatgggtga aaccccatct ctactaaaaa aatacaaaaa 42660
 ttattcggtt gtggtggcac acgcctgtaa tcccagctac ttgggaggct gaggcaggag 42720
 aatctcttga acctgggagg cagaggttgc agtgagtgga gatcccgcgc ttgcactcca 42780
 gcctgggcca cagagtgaga ctccatcaaa aaaaaaaaaa aaaaaaaaaa aaattagccg 42840
 ggcgtggttg cgtgcaccta tactccagc tacttgggag gctgaggcag gagaatcgct 42900
 tgaacctgga aggcggaggt cgagtgagc cgagatcgtg ccattgcact tcagcctggg 42960
 cgacagagcg agactctgtc tcaaaaataa taataataac aataactagc cgggcctggt 43020
 ggcacatgcc tgtagtccca gttactcagg aggcggaggc atgagactca ggtgaactag 43080
 ggagacagag gttgcagtga gccaaagatca caccactgca ctccagcctg gttgacagag 43140
 cgagactctg tctcaaaaaa aaaaaaatcc catttgctca ttttttgat actagtataa 43200
 ctatcactct aaaccagtta gtacttaaat caagcagata tgggagatgg tgaattacca 43260
 tctacagtgt tgcataatat gtcacatact gagcattatc agctagtaga atctagttaa 43320
 ttgttctatg tgtgatgtat gcagagttcc cattttgaat gtgtttttac tatgcttaaa 43380

-continued

taaatgactg atgtcagcaa ccccaaatg atacatctga tgaagagcc cctgttcccc	43440
aataataaca tctaaactat agacattgga atgaacaggt gccctaagt ttctccctc	43500
cagggtttct tggccggtct ctgaggacta cacatcccta ctcccgctt ttctcatctt	43560
caggcgagct aacagtatct ccaagtcccc tggccccagc tcccaaaagg agccctgct	43620
gttcagcgtg gacatcagcc gtcagaatc ccttcgttgt tccagcagct attcagagca	43680
gatcttccgg ccctgtgacc taatccatgg ggaggctctg gggaagggt tctttgggca	43740
ggctatcaag gtgagcgag gcaacaattg ctttgtctct ctgccccag tccctctgtc	43800
actgtcttct ggggatttct catcacttgg cccacccca caccatgcag gatgccaggc	43860
ctccttctct gctttgggtg ttggtgtgag aggtatcctt caccaccac caggccacct	43920
aaggtaaatg ttgctgttac agtgagcttg tggacctgga gatccaggtt ggggtgagct	43980
gtgcctgtgg ccctcctgcc tccagtcagt ggggtgttgt taggtgctg cagacctcag	44040
taccgggcat gctacaagga gcacacaggg gaatggctcc tgcctccctg gtgaacagtc	44100
tcagggacta acctctctct ttctctctc ctctctctct tctgctgaga actgggaggg	44160
ggggtcaggt aagacgtgtg tctcagcttg ggggcagcag ggctggagag ctacccccg	44220
atccaccag ctccctgttg catgtctttg gcactgacct tctgcccc agacttctgt	44280
tcactcagga gactcacttc tatgcaaat gaccagagcc cctgcttggc ttggcagcat	44340
ccctctctgc cttcttcccc acttcccttt tctgggttct tgcctgtcct ctgtcatgc	44400
ccagctctcc aggaagagg gtttgcttcc gtgtgagtc catgttgctc cagctgcat	44460
cttcacaca tgaactctgt cattctgacc cggctcagtg tgccctccaa gggatggat	44520
ggccagctgc atagatttct tcaaacagtt ctccagaact tctctgtgtc tcagcaccat	44580
taacagtcac cctccctgta ggtgacacac aaagccacgg gcaaatgat ggtcatgaa	44640
gagtaattc gatgtatga ggagaccag aaaactttc tgactgaggt aagaagatgg	44700
agggggcccg ggaggttgtt gtcaccattg gaagagagaa gaccttaca ataatggctt	44760
caagagaaaa tacagtttgg aattactgtc ttaaagacta agcagaaaag agccctagag	44820
gaatatccca ctccctctaa attacagctt aattatttgt tcaatgaaca cttactaaa	44880
gcaacacaaa cagggtacaa gggatgcagt aacaaaagat acagggttca gaagagctct	44940
cagggttatg ggaatgatga catgaaaca ctccaattta gtacaactca atgttataat	45000
cctcacctga acgcccctgt aaggagcctt ggaggggagc tccctgagca ctacactcc	45060
ttgggcattt acagttttca ctacccctcc caagttaact catggagtaa ctttaagttg	45120
ggacacctgt ggtctgggta ttgcccctca agccacttgg ccactccac cccagttctc	45180
ccaatgcagt tccaagggta aggcctatga agccatctcc atctatatgg tgggtgtctt	45240
ccctcatcct gatcttagtg ccctgtcata tcacaagata ggaggtagga gatacagtg	45300
gtaacacttg tcaagctgat tccctggagg gaagaggtaa ggaagacagt gagaagttaa	45360
ccaccagett tccctggctt ccccccccc caggtgaaag tgatgcgag cctggaccac	45420
cccaatgtgc tcaagttcat tgggtgtctg tacaaggata agaagctgaa cctgctgaca	45480
gagtacattg aggggggac actgaaggac tttctgcga gtatggtgag cacaccacc	45540
catagtctcc aggagccttg gtgggtgtg agacacctat gctatcacta cctaggagc	45600
ttaaagggca gaggggccct gctttgctc caaaggacca tgctgggtgg gactgagcat	45660
acatagggag gcttcaactg gagaccacat tgaccatgg ggctggacc acgagtgga	45720
cagggtcaa cagcctctga aaatattcc ccattctgca ggatccgttc cctggcagc	45780

-continued

agaaggtcag gtttgccaaa ggaatgcct ccggaatggt gagtcccacc acaaacctg 45840
 ccagcagggc gagagtaggg agaggtgtga gaattgtggg cttcactgga aggtagagac 45900
 cccctcctat gcaacttggt tgggctgggt cagcagctat tcattgagtt tgtctgtgtc 45960
 actgaaactg accccagcca actgttctca gttcacagcc ctgttttcaa agaattacac 46020
 atctctaaag gcaaacaggg caccgacaag gcaactgga gagggcaaac gtagcctgag 46080
 atggcctggg cttgccatca cagggtattca ggtgctgagg gcccttagac caactagagc 46140
 acctcactgc ctaggaaatc aatgaagggg aaatgagttc tagcggagcc ctgaaggatc 46200
 agaattggat aaagtctta ttggcagaga ggcaccagga ttgaagtgc aggagcaaa 46260
 acctgggagg aaagaggaga aaatcatcta ttccacctg aaacaaatga ttccaagcat 46320
 agaaataata acagctgaca agtactgagt gccctctata tgctaggcac tgggctgagg 46380
 gattaacatg catgtgcatg ttattctctc atgacaacct tggtttcag ataagctgga 46440
 ctggaaggg acagagctgg gatcctgggc taatcagtct ggtcgccaag cctgagactt 46500
 tagccactgc ccttcacatg ggggtccatg aaaatagtag tagtctggaa cagtttggg 46560
 gtacatcaag gtcgctgtgt tttaagctat ggagtctgga ctataggaga caaatgtaaa 46620
 agagttttt ggtgactgg ctttttggtt tttttgttg tttgtttgt ttgtttgtt 46680
 tttgtttgt ttttctgtt tctggggctt gaatcaggaa ggaggtttt ttgttgtgt 46740
 tgttttgaga aaggatattg ctctgttgcc cagactggag tgcagtggca cgatcatggc 46800
 tcaactacag ttcgacctcc tgggctcaag caatcctcct gcccttagcc cccaagtagc 46860
 tggactacag gtgtgtacca ccacacctaa ttttttgaat tttttttct tttttttt 46920
 tttttttt ggtagagaca ggttctcact ttgttgccca ggctgaatc tcaaacctc 46980
 gggctcaagc attcctcctg cctcgccctc ccaaagtgtt gggattacag ttgtgagcca 47040
 ccatgcccg caggaaaaga tttttaagca agaaagctta agagctgtgg tttttccaa 47100
 atgagtctg gctggcacag tggctcatgc ctgtaatccc agcactttt tgggaggccg 47160
 aggtgagtg atcacttgag gtcaggaggt tgagaccagc ctggccaact ggtgaaacc 47220
 ctgtttctac taaagaaaa aatgcaaaa ttagctgggc gtggtggtgc acgcctgtg 47280
 tccagctac tcaggaggcc gaggcaggag aatagcttga acctgggagg cagaagtgc 47340
 agtgagccaa gatcacacca ctgcattcca gccctgggtga cagagtga cttcatctca 47400
 aaaaaaaa aaagagaga ctgatatgt tagtacattg ggtggaatg cggagggtcc 47460
 agggaaatga gccctgcata gggggctaant gaaacatttc agatttctga attaaggtag 47520
 tggctgtggg gacaggagcc tgggaggcag ggtggagtca gaatggagag actggttggc 47580
 aatgaggga caggaggagg aggaggagga gttacgagt gcttgaggtg tcaactacca 47640
 gacatttggg ggtggggga tagccgtgat tgttgagcaa ctggtttggg aagagctagc 47700
 attgatccct gctgttctgt gctagcagaa cctatcagca tcttctgggc aggaactgg 47760
 ctccatgaga ctggcttagg gagaggctgc tagtcacctc atctgcagag aaggggcagc 47820
 tggagctgtg ggacagaaga ggcattccat tagctggtg ggtgtctca gcttgaag 47880
 aggagatggc tttgagcagg gctgacactg aaaaggctgg aagaaaaaa cagacacaca 47940
 agagtctcag gatcaggtag cataggaaa ttgtggacag tctttgagga gcaactccctc 48000
 aggcaggcag gcaggcaggt catgagctat agcgattcag gaagagctcc ctgggtgtgt 48060
 gagcagctcc aggagcctaa gggatgaaag tagtattgca gggggctgga gagcaaggag 48120

-continued

tggtccttc tacatttgca aggggaaggag aaaggaagtt gctcctgaga gtggttaagag 48180
 tcagtgttg aggcctggag agggagacata acaaacaaat ttgttgaca acattttggt 48240
 aggaagggg agagcttaaa gtttagacag tggggaagggt ggagtcctag aggaggtgaa 48300
 tgtctgaaag acagagctag ctggagcaag aagtcacttc tctgttgca gcaggaagga 48360
 tccaaagtgg ctcaagccag agattgggag agtggggagg agggagcagc ctggatctaa 48420
 gtaaatggg tagaggtgga ggggtgtctg caacggccag ggtttctga agtggggac 48480
 attagagag agctgtgagg gctttggcca gccactgtgc tagtgattgg tgaaccaaag 48540
 gatgggcagg agatggcagc aggggaagcag aggaagtcca ggcttctgt tggatttggg 48600
 acaagggaga ggccatagga ggcctggcc ctgtgtcca ggttgggttc tgaagctggg 48660
 tgggcatggc ctggtaggag agcatctatg gcgccaatt ccagattcag ggtctagttg 48720
 atttctggc cctgtagcct cagctcatgc ttctgttcca ggctatttg cactctatgt 48780
 gcatcatcca ccgggatctg aactgcaca actgcctcat caagttgga tgtccactg 48840
 ctctgggctt ggccctcagg gtcctatcct tcttggttc cttgtcaca aggaggctga 48900
 cttgtccctt ctggttagag ggcagaggtg ttgcctagga gctcctatct tcccttctt 48960
 gcttctcca atgcccttct ctgtcctctg ggagctccga gacacacaca gacataattt 49020
 cacttctct cattagcaac ctttgaata atttgattag aagggaacttc agaagtttgt 49080
 tgactatag tagaaaaacc tgcatttta cctgcttttg ccccatagta gtcttgaata 49140
 acagttcatt gctgacccca tttacagtg gtggacctg aagcctcagc ctgaggccac 49200
 cgagctagta aatttacagg gaccagtttg agaccagcat tccctccact gccctcagc 49260
 tgtgtgtgtt acaatgttgt ttgtcttact gacttctat ctggttctt ggtgtctac 49320
 cggctggccc tggctctgcc ctctagacc acaccacgca atcttcatc ctttccaca 49380
 tgactgccc gtactatc aaagagcttg tctcccca gtctcccat ctactgctc 49440
 cacttgctt ttttctgt tctctgtgt ctgacctg cctgaatca ttttaggaat 49500
 aagacaggac agggaaaaac aaagcaacc ccctgtcca cctctgagt ccactctcca 49560
 agtccctgag cctcacctcc agggctccag tggctctgcc atgaaccac tgtgggtgtg 49620
 gagtctgctg tgcacagata ccagaccctc agaaacaca atgcaagtg tgtctgttt 49680
 tttgtttgt tttgtttgt ttttagatg gagtctcatt ctgttcca ggctggagt 49740
 cagtgtgca atcttgctt actgcagct ctacctccc ggttctagt attgtctgc 49800
 ttcagctcc cagttagtag gactacaggc gtgtgccacc acgcccagct aattttttt 49860
 tttttttt tgtattttta gttagacag ggttttgcca tgttgccag gctgtctt 49920
 aactctgac ctcaagtgat tccccgctt tggcctcca aagttctgg attacagtg 49980
 gaagccacc tgcctggct gagtgtgtt atttgataga gtttctgt ctgattctcc 50040
 cttgtatac acctttctt ccttctcag tggcttctt tgcctatgt tcttccca 50100
 ggccaggtt gagaacatcc ccatgaagtc ctgacctgtc ttttatcta ccaggacaag 50160
 actgtgtgtg tggcagactt tgggtgtca cggctcatag tggagagag gaaaaggcc 50220
 cccatggaga aggccaccac caagaaacgc accttgcca agaacgacc caagaagcg 50280
 tacacgtgtg tgggaaacc ctactggatg gccctgaga tgetgaacg tgaatctga 50340
 agcctggag gggacaccg cagaggagg acagatgtc ccttgcatc agagccctg 50400
 gaattccagg ggaggcctgt gaagcgtag accgatacc cagagctgag gatattttt 50460
 ccttgccagg tggggcctc cgatttagct cctgagctca gggggctgg aactgatcag 50520

-continued

tgtcccatca tgggggataa ggtgagttct gactgtggca tttgtgcctc agggatcgct 50580
 aagagctcag gctattgtcc cagcttttagc cttctctctc catggtgaga actgaagtgt 50640
 ggtgccctct ggtggataat gctcaaacca accagagatg ctggttgga ttcttgaaat 50700
 cagggttggt aggcctcaga aatgggtctga atacaatcca ttttgagtc tagggccag 50760
 agaagtccag tgaattgcct aggcagcacc agctgcctaa tggcagaggg tagatgaacc 50820
 ctagctctggt tcttttccac ttaaacgtgc agtttcatcc taggcagtgt tatgttataa 50880
 gggctctcca aggcagttca cctacggctg aggaaggact attttcaggt ggtgtctgcg 50940
 caggacagcc tgtgggggtg ccctacagaa cctgttctag ccctagtctt tagctgtggc 51000
 ttagattgac cctagaccca gtgcagagca ggtaagggat gtaaacctaa cagtgtgctc 51060
 tcctgtgttc cccaaggaaa gagctatgat gagacggtgg atatctctc ctttgggac 51120
 gttctctgtg aggtgagctc tggcaccaag gccatgccc aggcagcagg cctagcagct 51180
 ctgccttccc tcggaactgg ggcactcct cctagggatg actagcttga ctaaatcaa 51240
 catgggtgta ggggtttatg gtttataacg catctgcaca tctttgccac gttcgtgttt 51300
 cattggtctt aagagaagga ctggcagggt tttttgttt tagatggagc ctcacttctg 51360
 tgcccaggct ggagtgcagt ggcacaatct gggctcactg caacctctgc cttctgggtt 51420
 caagtattc tcctgcctca gccctccaa tagctgggac taccggcaca caccaccatg 51480
 cccggctaatt tttgtattt ttagtagaga cagggtttca ccatgttggc caggctggtc 51540
 ttgaactccg gacctcaggt gatccgctg cctcagcctc taaaagtgt ggaattaata 51600
 ggctgtgact acctcgccc ggcagggttt ttttttttt tttttagtg aggaactga 51660
 ggcttggaag agggcagtggt cttgcacatg gtcgataagg ggcagatgag actcagaatt 51720
 ccagaaggaa gggcaagaga ctgttcattg ggctgtctag ctagctcttg ggccaatgt 51780
 agcccttctc agttccctc aagtagaagt agccactcta ggaagtgtca gccctgtgcc 51840
 aggtaccacg tggacagagt gaggaatctt ggaaagattc ctaccttag gagttagtc 51900
 aggtgacagc atatctcagc gactcaaaca cacacacatt caaagccttc tgtaattcct 51960
 acaaagttgt gagggttaga ggagaggaga gacaagggat ggttaggata atgaaggat 52020
 gttttgtttt tgtttttgtt ttgagatgg agtttctc tgtcaccag gctggagtgc 52080
 agaggtgcaa tcttggtcca ctgcagcctc cgctcccag gttcaagca tcctcctgcc 52140
 tcagcctccc aagtagctgg gactacaggt gtgcgccacc acgcttggt aatttttga 52200
 ttttcagtag agacagggtt tcgcatatt ggccaggctg gtctcaaatg cctgacctca 52260
 ggtgatacac ccgttcagc ctcccaagt gctgagatta caggcatgag ctaccgtgcc 52320
 tggccatgaa ggaagatttg ttttaaaaa ttgttttctt taatattaat tgaacacctc 52380
 tgttcagagc actgggctgg tgccagaggg tttcagacat gaatcagatc cagcacctca 52440
 tagagcctta atctggcaca cacacacag cacaaggaga cacagacaag gcagggtagg 52500
 atgagtggaa gctaggagca gatctgatt tggaacactt ggcttctgca gtgaagcccc 52560
 ttcttagtcc tcttcagtaa ccagctctc agtggtatca ggtctgatt agtaagattt 52620
 ggagagatga ttggggattg gggagagctc tctaacctat tttaccacct cctctctgc 52680
 cattcttctc gtccacatcc ccagcatccc tttcccttgc caagtatctg tggcctctgt 52740
 agtcctttgt aaacagctgt cttcttacc tacagatcat tgggcagtg tatgcagatc 52800
 ctgactgcct tccccgaaca ctggactttg gctcaacgt gaagctttt tgggagaagt 52860

-continued

ttgttcccc agattgtccc cggccttct tcccgctggc cggcatctgc tgcagactgg 52920
agcctgagag caggttggta tcttgccttt ttctccagc tcacaggggc ctgggacgtt 52980
tgccctctgtc taaggccacc cctgagccct ctgcaagcac aggggtgaga gaagccttga 53040
ggtcaagaat gtggctgtca acccctgagc catctgacaa cacatatgta caggttggag 53100
aagagagagg taaagacata gcagcaagta atctggatag gacacagaaa cacagccatt 53160
aaaagaaagt ttaaagaag gaaattcacc caaaccattt gaatacagta agtgatttca 53220
tctttcgata ttccctgtc catatctaca catatacttt tttttatagt aaatagtctt 53280
gtattttgcc ctgcatttcc cttgtgttta ctatccagtc ttctgttta tcatttttgt 53340
cgacaacatg aaattctatt gagagactgt ctgaacatat tgtaatgtag atgttcaggt 53400
ttttccagtt tctctttaca ataggtattt aactacagtg agcagtttta tgcatttagc 53460
taatttctcc tttaggaag tattttcaaa attacctta ttcttctcag gtaataattt 53520
cattattacc aaagttacc taggtcttt caagtgtgtg gttaaaaaac gagaatctgg 53580
ctgggcgcga tggctcacc ctgtaatccc agcactttgg gaggtgagg ctggtggatc 53640
acctgaggtc tggagttcga gaccagcctg gccaacatgg tgaacccca tctctactaa 53700
aaatacaaaa cttagccagg catggtggca ggtgcctgta accccagcta ctggggaggc 53760
tgaggcagga gaattgctt aaccagggg cggaggttgc agtgagccga tatcacgcca 53820
ttgcactcca gcctcggcaa caagagtga actctgtctc aaaaatgggg ttcttttctt 53880
gccatcaaaa atcatgtttc ttttaaaac aagttcaaac attaccaag tttatagcac 53940
aggaaatagc tcttctgtaa tctcccttaa ccaatatatc cctcaacatt ctctcacc 54000
ccaactccac cctcccagga taaccagttg ggacataatc tttattttaa aatggtttcc 54060
ggatagagaa agcgtctcgg cggcggcagc cccggcgcg cccgcagggg acaagggcg 54120
ggcggtacgg cggggagggg cggggcgcg accagggcag gcccgggggc tccgcatgct 54180
gcagctgcct ctggggcgcc cccgcgcgc cctcgccgc ggagccggcg agctaacctg 54240
agccagccgg cggcgctcag ggagggcgcg gcacaaggag gggcccccag cgcgcacgtg 54300
gccccggagg ccgcctgtgc ggacagcggc accgcggggg gcgcggcggt ggcggccccg 54360
gccccggccc ccaggccagg cagtggcggc caaggaccac gcattctact tcagagcccc 54420
ccccggggcc gcaggagagg gcccgggctg ggcggatgat gagggcccag tgaggcgcca 54480
agggaggtc acctcaagt atgaccccaa ggagctacgg aagcacctca acctagagga 54540
gtggatcctg gagcagctca cgcgcctcta cgaactgccag gaagaggaga tctcagaact 54600
agagattgac gtggatgagc tcttgacat ggagagtgc gatgcctggg ctccaggggt 54660
caaggagctg ctggttgact gttacaaacc cacagaggcc ttcattctg gcctgctgga 54720
caagatccgg gccatgcaga agctgagcac accccagaag aagtgagggt ccccgaccca 54780
ggcgaacggt ggctccata ggacaatgc taccgccga cctcgtagca acagcaatac 54840
cgggggaccc tgcggccagg cctggttcca tgagcagggc tctcgtgcc cctggcccag 54900
gggtctctc cctgcccc tcagttttcc acttttggat tttttattg ttattaaact 54960
gatgggactt tgtgtttta tattgactct gcggcacggg cccttaata aagcgaggta 55020
gggtacgctt ttgtgcagc tcaaaaaaa aaaaaaaat gatttccagc ggtccacatt 55080
agagttgaaa ttttctggg ggagaatcta taccttgttc ctttataggc caaggaccgc 55140
agtccttcag taacaccagt gtaaaagctt gaggagaaat tgtgaagcta cacagtattt 55200
gttttcta acctctgtc attctaata tctttaattt attaaaaat atatataac 55260

-continued

agtattgaat gcctactgtg tgctaggtac agttcctaac acttgggtta cagcagcgaa 55320
 caaaataaag gtgcttacc tcatagaaca tagattctag catggtatct actgtatcat 55380
 acagtagata caataagtaa actatattga atattagaat gtggcagatg ctatggaaaa 55440
 agagtcaaga caagtaaaga cgattgttca ggggtaccagt tgcaatttta aatatggctg 55500
 tcagagcagg cctcactgag gtgacatgac atttaagcat aaacatggag gaggaggagt 55560
 aagcctgagc tgtcttaggc ttccggggca gccaaagccat ttccgtggca ctaggagcct 55620
 ggtgtttccg attccacctt tgataactgc attttctcta agatatggga gggaagtgtt 55680
 tctcctattg ttttaagta ttaactccag ctagtccagc cttgttatag tgttacctaa 55740
 tctttatagc aaatatatga ggtaccggta acattatgcc catttctcac agaggcacta 55800
 ctagggtgaag gagtgtgcct gacgttatac aaccaggaag tagctgagcc tagatccctt 55860
 ccacccccc catggccctg ctcatgttcc acctgcctct aattacctc ttttcttct 55920
 agaccagcat tctcgaatt ggaggactcc tttgagcccc tctccctgta cctgggggag 55980
 ctgggcaccc cgctgcctgc agagctggag gagtgggacc aactgtgag catgcagtac 56040
 ggccctgaccc gggactcacc tccctagccc tggcccagcc cctcgaggg ggtgttcta 56100
 cagccagcat tgccctctg tgccccattc ctgctgtgag cagggccgctc cgggcttct 56160
 gtggattggc ggaatgttta gaagcagaac aagccattcc tattacctcc ccaggaggca 56220
 agtgggcgca gcaccaggga aatgtatctc cacaggttct ggggcctagt tactgtctgt 56280
 aaatccaata cttgcctgaa agctgtgaag aagaaaaaa cccctggcct ttgggccagg 56340
 aggaatctgt tactcgaac caaccaggaa ctccctggca gtggattgtg ggaggctctt 56400
 gcttaccta atcagcgtga cctggacctg ctgggcagga tcccagggtg aacctgcctg 56460
 tgaactctga agtactagt ccagctgggt gcaggaggac ttcaagtgtg tggacgaaag 56520
 aaagactgat ggctcaaagg gtgtgaaaaa gtcagtgtg ctccccctt ctactccaga 56580
 tcctgtcctt cctggagcaa ggttgaggga gtaggtttt aagagtcctt taatatgtg 56640
 tggaaacagg caggagttag aaaaagggtt ggcttctgtt tacctgtca ctggctctag 56700
 ccagcccagg gaccacatca atgtgagagg aagcctccac ctcatgtttt caaactaat 56760
 actggagact ggctgagaac ttacggacaa catcctttct gtctgaaaca aacagtcaca 56820
 agcacaggaa gaggctggg gactagaaag aggcctgcc ctctagaaag ctcatctt 56880
 ggcttctgtt actcactc gggtgggctc cttagttaga tgcctaaaac attttgccta 56940
 aagctcagtg ggttctggag gacagtgtg cttgtcacag gcctagagtc tgaggaggag 57000
 gagtgggagt ctacgaatc tcttggctt ggcttcatg caaccactgc tcaccttca 57060
 acatgcctg tttaggcagc agcttgggtt gggaagaggt ggtggcagag tctcaagct 57120
 gagatgctga gagagatagc tccctgagct gggccatctg acttctacct ccatgtttg 57180
 ctctcccaac tcattagctc ctgggcagca tccctctgag ccacatgtgc aggtactgga 57240
 aaacctccat cttggctccc agagctctag gaactcttca tcacaactag atttgcctct 57300
 tctaagtgtc tatgagcttg caccatattt aataaattgg gaatgggttt ggggtattaa 57360
 tgcaatgtgt ggtgtgtgta ttggagcagg ggaattgat aaaggagagt ggtgtctgtt 57420
 aatattatct tatctattgg gtgttatgtg aaatattgta catagacctg atgagtgtg 57480
 ggaccagatg tcactctctg tcagagttta cttgctatat agactgtact tatgtgtgaa 57540
 gtttgcaagc ttgctttagg gctgagccct ggactccag cagcagcaca gttcagcatt 57600

-continued

gtgtggctgg ttgtttcctg gctgtcccca gcaagtgtag gagtgggtgg cctgaactgg 57660
 gccattgac agactaata aattaagcag ttaacataac tggcaatatg gagagtga 57720
 acatgattgg ctcagggaca taaatgtaga gggctgtgta gccaccttct ggcctagccc 57780
 acacaaactc cccatagcag agagttttca tgcacccaag tctaaaacc tcaagcagac 57840
 acccatctgc tctagagaat atgtacatcc cactgagggc agccccttcc ttgcagcagg 57900
 tgtgactgac tatgacctt tctgtgctg gctctcacat gccagctgag tcatctctta 57960
 ggagccctac cctttcatcc tctctatatg aatacttcca tagcctgggt atcctggctt 58020
 gctttctca gtgtgggtg ccacctttgc aatgggaaga aatgaatgca agtcaccca 58080
 ccccttgtgt ttccttaca gtgttgaga ggagaagacc agtttcttct tgctttgca 58140
 tgtgggggat gtcgtagaag agtgaccatt gggaaggaca atgctatctg gttagtggg 58200
 cctggggc aatataaato tgtaaaccca aaggtgtttt ctcccaggca ctctcaagc 58260
 ttgaagaatc caacttaagg acagaatatg gttcccgaaa aaaactgatg atctggagta 58320
 cgcattgctg gcagaaccac agagcaatgg ctgggcatgg gcagaggta tctgggtgtt 58380
 cctgaggctg ataacctgtg gctgaaatcc ctgtctaaaa gtccaggaga cactcctgtt 58440
 ggtatctttt cttctggagt catagtagtc acctgcagg gaacttctc agcccagggc 58500
 tgctgcaggc agcccagtga cccttctcc tctgcagtta tcccccttt ggtgtgtgca 58560
 gcaccacccc cgtcacccac caccacaacc ctgcccgaact ccagccttta acaagggtg 58620
 tctagatatt cattttaact acctccacct tggaaacaat tgtgaaggg gagaggattt 58680
 gcaatgacca accaccttgt tgggacgct gcacacctgt ctttctgtct tcaacctgaa 58740
 agattcctga tgatgataat ctggacacag aagccgggca cggtggctct agcctgta 58800
 ctcagcactt tgggaggcct cagcagggtg atcacctgag atcaagagtt tgagaacagc 58860
 ctgaccaaca tgggaaacc ccgtctctac taaaaataca aaaattagcc aggtgtggtg 58920
 gcacatacct gtaatccag ctactctgga ggctgaggca ggagaatgc ttgaaccac 58980
 aaggcagagg ttgcagtgag gcgagatcat gccattgcac tccagcctgt gcaacaagag 59040
 ccaaaactcca tctcaaaaa aaaaa 59065

<210> SEQ ID NO 4
 <211> LENGTH: 265
 <212> TYPE: PRT
 <213> ORGANISM: Human

<400> SEQUENCE: 4

Leu Thr Glu Val Lys Val Met Arg Ser Leu Asp His Pro Asn Val Leu
 1 5 10 15
 Lys Phe Ile Gly Val Leu Tyr Lys Asp Lys Lys Leu Asn Leu Leu Thr
 20 25 30
 Glu Tyr Ile Glu Gly Gly Thr Leu Lys Asp Phe Leu Arg Ser Met Asp
 35 40 45
 Pro Phe Pro Trp Gln Gln Lys Val Arg Phe Ala Lys Gly Ile Ala Ser
 50 55 60
 Gly Met Ala Tyr Leu His Ser Met Cys Ile Ile His Arg Asp Leu Asn
 65 70 75 80
 Ser His Asn Cys Leu Ile Lys Leu Asp Lys Thr Val Val Val Ala Asp
 85 90 95
 Phe Gly Leu Ser Arg Leu Ile Val Glu Glu Arg Lys Arg Ala Pro Met
 100 105 110

-continued

Glu	Lys	Ala	Thr	Thr	Lys	Lys	Arg	Thr	Leu	Arg	Lys	Asn	Asp	Arg	Lys	
	115						120					125				
Lys	Arg	Tyr	Thr	Val	Val	Gly	Asn	Pro	Tyr	Trp	Met	Ala	Pro	Glu	Met	
	130					135					140					
Leu	Asn	Gly	Lys	Ser	Tyr	Asp	Glu	Thr	Val	Asp	Ile	Phe	Ser	Phe	Gly	
	145				150					155					160	
Ile	Val	Leu	Cys	Glu	Ile	Ile	Gly	Gln	Val	Tyr	Ala	Asp	Pro	Asp	Cys	
			165					170						175		
Leu	Pro	Arg	Thr	Leu	Asp	Phe	Gly	Leu	Asn	Val	Lys	Leu	Phe	Trp	Glu	
		180						185					190			
Lys	Phe	Val	Pro	Thr	Asp	Cys	Pro	Pro	Ala	Phe	Phe	Pro	Leu	Ala	Ala	
		195					200					205				
Ile	Cys	Cys	Arg	Leu	Glu	Pro	Glu	Ser	Arg	Pro	Ala	Phe	Ser	Lys	Leu	
	210				215					220						
Glu	Asp	Ser	Phe	Glu	Ala	Leu	Ser	Leu	Tyr	Leu	Gly	Glu	Leu	Gly	Ile	
	225			230					235					240		
Pro	Leu	Pro	Ala	Glu	Leu	Glu	Glu	Leu	Asp	His	Thr	Val	Ser	Met	Gln	
			245					250						255		
Tyr	Gly	Leu	Thr	Arg	Asp	Ser	Pro	Pro								
		260					265									

That which is claimed is:

1. An isolated nucleic acid molecule consisting of a nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence that encodes an amino acid sequence shown in SEQ ID NO:2;
- (b) a nucleic acid molecule consisting of the nucleic acid sequence of SEQ ID NO:1;
- (c) a nucleic acid molecule consisting of the nucleic acid sequence of SEQ ID NO:3; and
- (d) a nucleotide sequence that is completely complementary to a nucleotide sequence of (a)-(c).

2. A nucleic acid vector comprising a nucleic acid molecule of claim 1.

3. A host cell containing the vector of claim 2.

4. A process for producing a polypeptide comprising culturing the host cell of claim 3 under conditions sufficient for the production of said polypeptide, and recovering the peptide from the host cell culture.

5. An isolated polynucleotide consisting of a nucleotide sequence set forth in SEQ ID NO:1.

6. An isolated polynucleotide consisting of a nucleotide sequence set forth in SEQ ID NO:3.

7. A vector according to claim 2, wherein said vector is selected from the group consisting of a plasmid, virus, and bacteriophage.

8. A vector according to claim 2, wherein said isolated nucleic acid molecule is inserted into said vector in proper orientation and correct reading frame such that the protein of SEQ ID NO:2 may be expressed by a cell transformed with said vector.

9. A vector according to claim 8, wherein said isolated nucleic acid molecule is operatively linked to a promoter sequence.

* * * * *

P

This Page Blank (uspto)



US006531309B1

(12) **United States Patent**
Hu et al.

(10) Patent No.: **US 6,531,309 B1**
(45) Date of Patent: **Mar. 11, 2003**

(54) **HUMAN TRANSPORTER PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME**

(75) Inventors: **Yi Hu**, The Woodlands, TX (US);
James Alvin Kleke, Houston, TX (US);
Brian Zambrowicz, The Woodlands, TX (US); **Jean-Pierre Revelli**, Spring, TX (US)

(73) Assignee: **Lexicon Genetics Incorporated**, The Woodlands, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/795,927**

(22) Filed: **Feb. 28, 2001**

Related U.S. Application Data

(60) Provisional application No. 60/185,956, filed on Feb. 29, 2000.

(51) Int. Cl.⁷ **C07H 21/04**; C12N 15/63;
C12N 1/21; C12N 1/15; C12N 15/85; C12N 15/86

(52) U.S. Cl. **435/252.3**; 435/320.1;
435/254.11; 435/254.2; 435/325; 536/23.5

(58) Field of Search 536/23.5, 23.4,
536/23.2, 23.1; 530/350; 435/320.1, 325,
252.3, 254.11, 254.2

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,215,051 A	7/1980	Schroeder et al.
4,376,110 A	3/1983	David et al.
4,594,595 A	6/1986	Struckman
4,631,211 A	12/1986	Houghten
4,689,405 A	8/1987	Frank et al.
4,713,326 A	12/1987	Dattagupta et al.
4,946,778 A	8/1990	Ladner et al.
5,198,344 A	3/1993	Croop et al.
5,252,743 A	10/1993	Barrett et al.
5,424,186 A	6/1995	Fodor et al.
5,445,934 A	8/1995	Fodor et al.
5,459,127 A	10/1995	Felgner et al.
5,556,752 A	9/1996	Lockhart et al.
5,658,782 A	8/1997	Amara et al.
5,700,637 A	12/1997	Southern
5,744,305 A	4/1998	Fodor et al.
5,837,458 A	11/1998	Minshull et al.
5,866,699 A	2/1999	Smyth
5,869,336 A	2/1999	Meyer et al.
5,877,397 A	3/1999	Lonberg et al.
5,948,767 A	9/1999	Scheule et al.
6,075,181 A	6/2000	Kucherlapati et al.
6,110,490 A	8/2000	Thierry
6,150,584 A	11/2000	Kucherlapati et al.

FOREIGN PATENT DOCUMENTS

JP	11-146790	6/1999
WO	WO 90/06047 A2	6/1990
WO	WO 98/44954 A1	10/1998
WO	WO 98/55858 A1	12/1998

OTHER PUBLICATIONS

Lohi et al, Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. Genomics 70:102-112, 2000.*

Peer Bork and Eugene V. Koonin, Predicting functions from protein sequences—where are the bottlenecks? Nature Genetics 18:313-318, 1998.*

Ji et al, G-protein-coupled receptors, J. Biol. Chem, 273:17299-17302, 1998.*

Bird et al, 1988, "Single-Chain Antigen-Binding Proteins", Science 242:423-426.

Bitter et al, 1987, "Expression and Secretion Vectors for Yeast", Methods in Enzymology 153:516-544.

Colbere-Garapin et al, 1981, "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol. 150:1-14.

Gautier et al, 1987, "α-DNA IV: α-anomeric and β-anomeric tetrathymidylates covalently linked to intercalating oxazolopyridocarbazole. Synthesis, physicochemical properties and poly (rA) binding", Nucleic Acids Research 15(16):6625-6641.

Greenspan et al, 1993, "Idiotypes: structure and immunogenicity", FASEB Journal 7:437-444.

Huse et al, 1989, "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", Science 246:1275-1281.

Huston et al, 1988, "Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*", Proc. Natl. Acad. Sci. USA 85:5879-5883.

Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and R Nase H", FEBS Letters 215(2):327-330.

Inoue et al, 1987, "Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides", Nucleic Acids Research 15(15):6131-6149.

Inouye & Inouye, 1985, "Up-promoter mutations in the lpp gene of *Escherichia coli*", Nucleic Acids Research 13(9):3101-3110.

Janknecht et al, 1991, "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus", PNAS 88:8972-8976.

Kohler & Milstein, 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity", Nature 256:495-497.

Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection", Proc. Natl. Acad. Sci. USA 81:3655-3659.

(List continued on next page.)

Primary Examiner—Elizabeth Kemmerer

Assistant Examiner—Ruixiang Li

(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are disclosed that can be used in therapeutic, diagnostic, and pharmacogenomic applications.

4 Claims, No Drawings

OTHER PUBLICATIONS

- Lowy et al, 1980, "Isolation of Transforming DNA: Cloning the Hamster apt Gene", Cell 22:817-823.
- Morrison et al, 1984, "Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains", Proc. Natl. Acad. Sci. USA 81:6851-6855.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", Proc. Natl. Acad. Sci. USA 78(4):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies possessing novel effector functions", Nature 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", J. of Immunology 147:2429-2438.
- Ruther et al, 1983, "Easy identification of cDNA clones", EMBO Journal 2(10):1791-1794.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", Gene 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", Proc. Natl. Acad. Sci. USA 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", J. Virol. 46(2):584-593.
- Stein et al, 1988, "Physicochemical properties of phosphorothioate oligodeoxynucleotides", Nucleic Acids Research 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", Proc. Natl. Acad. Sci. USA 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", Nature 314:452-454.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", J. Biol. Chemistry 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", Cell 11:223-232.
- Japanese Abstract, JP 11-146790, XP002177829.
- Nagase et al., "Prediction of the Coding Sequences of Unidentified Human Genes, XIII. The Complete Sequences of 100 New cDNA Clones from Brain Which Code for Large Proteins in vitro," DNA Research, Universal Academy Press, JP, vol. 6, 1999, pp. 63-70, XP000952912.
- Database EMBL Sequence Database (online), Hinxton, UK, Apr. 9, 1999, O'Hara et al., "*Homo sapiens* mRNA for KIAA0956 Protein, Partial cds.," XP002177825; EMBL:AB023173; abstract.
- Halleck et al., "Differential Expression of Putative Transbilayer Amphipath Transporters," Physiological Genomics, vol. 1, 1999, pp. 139-150, XP002177823.
- Database EMBL Sequence Database (Online), Hinxton, UK; Nov. 24, 1999, Halleck et al., "*Homo sapiens* putative E1-E2 ATPase mRNA, partial cds.," XP002177826, EMBL:AF15648; abstract.
- Database EMBL Sequence Database (Online), Hinxton, UK; Nov. 17, 1999, Bloecker et al., "*Homo sapiens* mRNA; cDNA DKFZp434N1615 (from clone DKFZp434N1615); partial cds.," XP002177827; EMBL:HSM801332, Accession No. AL133061; abstract.
- Database EMBL Sequence Database (Online), Hinxton, UK; Mar. 12, 1999, Ottenwaelder et al., "*Homo sapiens* EST DKFZp434J238_r1 (from clone DKFZp434J238_r1) EST partial cds.," XP002177828, EMBL:HSM011423, Accession No. AL046573; abstract.
- McMahon et al., "Mammalian Zinc Transporters," Journal of Nutrition, vol. 128, No. 4, Apr. 1998, pp. 667-670, XP002177824; ISSN: 0022-3166.
- International Search Report, International Application No. PCT/US01/06462, Sep. 26, 2001 (Attorney Docket No. LEX-0141-PCT).

* cited by examiner

HUMAN TRANSPORTER PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application No. 60/185,956 which was filed on Feb. 29, 2000 and is herein incorporated by reference in its entirety.

INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins that share sequence similarity with mammalian transporter proteins. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed polynucleotides, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed polynucleotides that can be used for diagnosis, drug screening, clinical trial monitoring, and treatment of diseases and disorders.

BACKGROUND OF THE INVENTION

Transporter proteins are integral membrane proteins that mediate or facilitate the passage of materials across the lipid bilayer. Given that the transport of materials across the membrane can play an important physiological role, transporter proteins are good drug targets. Additionally, one of the mechanisms of drug resistance involves diseased cells using cellular transporter systems to export chemotherapeutic agents from the cell. Such mechanisms are particularly relevant to cells manifesting resistance to a multiplicity of drugs.

SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins, and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPS) described for the first time herein share structural similarity with mammalian ion transporters, calcium transporters (particularly calcium transporting ATPases), sulfate transporters, and zinc transporters.

The novel human nucleic acid sequences described herein, encode alternative proteins/open reading frames (ORFS) of 1,177 and 374 amino acids in length (calcium-transporting ATPase, SEQ ID NOS: 2 and 4), 970 (sulfate transporter, SEQ ID NO:7), and 507 (zinc transporter, SEQ ID NO:10) amino acids in length.

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof, that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHP polynucleotides (e.g., expression constructs that place the described polynucleotide under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" (which can be conditional) that do not express a functional NHP. Knock-out mice can

be produced in several ways, one of which involves the use of mouse embryonic stem cells ("ES cells") lines that contain gene trap mutations in a murine homolog of at least one of the described NHPs. When the unique NHP sequences described in SEQ ID NOS:1-11 are "knocked-out" they provide a method of identifying phenotypic expression of the particular gene as well as a method of assigning function to previously unknown genes. Additionally, the unique NHP sequences described in SEQ ID NOS:1-11 are useful for the identification of coding sequence and the mapping a unique gene to a particular chromosome.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequences of the described NHP ORFs that encode the described NHP amino acid sequences. SEQ ID NOS 5, 8, and 11 describe nucleotides encoding NHP ORFs along with regions of flanking sequence.

DETAILED DESCRIPTION OF THE INVENTION

The NHPs described for the first time herein are novel proteins that may be expressed in, inter alia, human cell lines, fetal brain, pituitary, cerebellum, thymus, spleen, lymph node, bone marrow, trachea, kidney, fetal liver, liver, prostate, testis, thyroid, adrenal gland, salivary gland, stomach, small intestine, colon, adipose, rectum, pericardium, bone marrow, placenta, and gene trapped human cells. More particularly, the NHP that is similar to sulfate transporters (and the down-regulated in adenoma, or DRA, gene) is predominantly found in bone marrow and testis, and the zinc transporter-like NHP can be found expressed in the placenta.

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described polynucleotides, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of the NHPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal (or hydrophobic transmembrane) sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of an NHP, or one of its domains (e.g., a receptor or ligand binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, anti-

sense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of a DNA sequence that encodes and expresses an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, supra), yet still encodes a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP nucleotide sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-11 can be used as a hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics, polymers, metal or metallized substrates, crystalline or polycrystalline substrates, etc.). Of particular note are spatially addressable arrays (i.e., gene chips, microtiter plates, etc.) of

oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-11, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, inter alia, U.S. Pat. Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-11 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-11.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-11 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

Probes consisting of sequences first disclosed in SEQ ID NOS:1-11 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

As an example of utility, the sequences first disclosed in SEQ ID NOS:1-11 can be utilized in microarrays or other assay formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences first disclosed in SEQ ID NOS:1-11 in silico and by comparing previously collected genetic databases and the disclosed sequences using computer software known to those in the art.

Thus the sequences first disclosed in SEQ ID NOS:1-11 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific oligonucleotide sequence(s) first disclosed in the SEQ ID NOS: 1-11. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (e.g., the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, Mich., etc.), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a

phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Bioscience, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene.

The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, connective tissue disorders, infertility, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue can be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, N.Y.).

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869, 336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of the NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor for an NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP receptor. Nucleotide con-

structs encoding such NHP products can be used to genetically engineer host cells to express such products in vivo; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

The NHP Sequences

The cDNA sequences and the corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing. The NHP nucleotides were obtained from clustered human gene trapped sequences, testis and mammary transcript RACE products, ESTs, and human brain, testis, trachea, pituitary, thymus, and mammary gland cDNA libraries (Edge Biosystems, Gaithersburg, Md.).

SEQ ID NOS:1-5 describe sequences that are similar to eucaryotic ATP-driven ion pumps such as calcium transporting ATPases, and which can be found expressed in a variety of human cells and tissues. The described sequences were assembled using gene trapped sequences and clones isolated from human kidney, lymph node, and thymus cDNA libraries (Edge Biosystems, Gaithersburg, Md.).

SEQ ID NOS:6-8 describe sequences that are similar to, inter alia, sulfate transporter and cotransporter proteins, and can be found expressed in human bone marrow and testis. Several polymorphisms were found in this NHP including, but not limited to, possible A-to-G transitions at nucleotide positions corresponding to nucleotides 589, 692, 917, 1,164, and 2,390 of, for example SEQ ID NO:8 which be silent or can result in the met corresponding to amino acid position 73 of SEQ ID NO:7 converting to a val (e.g., met 73 converting to val 73), val 148 converting to ile, asn 230 converting to lys, ile 562 converting to val. An additional C-to-T transition was identified that converts ala 777 to val. SEQ ID NOS:6-8 can be expressed in bone marrow and predominantly in testis cells. These NHPs were assembled from gene trapped sequences and clones from a human testis cDNA library (Edge Biosystems, Gaithersburg, Md.).

SEQ ID NOS:9-11 describe sequences that are similar to zinc transporters and vesicular transporters, can be found expressed in, inter alia, placenta and adrenal gland, and these NHP sequences were assembled using gene trapped sequences and clones from human adrenal and placenta cDNA libraries (Edge Biosystems, Gaithersburg, Md.).

Transporters and transporter related multidrug resistance (MDR) sequences, as well as uses and applications that are germane to the described NHPs, are described in U.S. Pat. Nos. 5,198,344 and 5,866,699 which are herein incorporated by reference in their entirety.

NHPS and NHP Polypeptides

NHPs, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include but are not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to a NHP, as reagents in assays for screening for compounds that can be as pharmaceutical

reagents useful in the therapeutic treatment of mental, biological, or medical disorders and diseases. Given the similarity information and expression data, the described NHPs can be targeted (by drugs, oligos, antibodies, etc.) in order to treat disease, or to therapeutically augment the efficacy of, for example, chemotherapeutic agents used in the treatment of breast or prostate cancer.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP polynucleotides. The NHPs typically display have initiator methionines in DNA sequence contexts consistent with a translation initiation site.

The NHP amino acid sequences of the invention include the amino acid sequence presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a substrate of a NHP, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where, as in the present instance, the NHP peptide or polypeptide is thought to be membrane protein, the hydrophobic regions of the protein can be excised and the resulting soluble peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, in situ. Purification or enrichment of a NHP from

such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Pharmacia or American Type Culture Collection) can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted sequence is expressed (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus

is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase

(Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp⁺ or apr⁺ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg⁺, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Also encompassed by the present invention are fusion proteins that direct the NHP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHPs to antibody molecules or their Fab fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHP would also transport the NHP to the desired location within the cell. Alternatively targeting of NHP or its nucleic acid sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in *Liposomes: A Practical Approach*, New RRC ed., Oxford University Press, New York and in U.S. Pat. Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective disclosures which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHP to the target site or desired organ. This goal may be achieved by coupling of the NHP to a cytokine or other ligand that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. application Ser. Nos. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes if needed and can optionally be engineered to include nuclear localization sequences when desired.

Antibodies to NHP Products

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab)₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of NHP in a biological sample and may,

therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction with gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with a NHP, an NHP peptide (e.g., one corresponding to a functional domain of an NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Pat. Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety. Also encompassed by the present invention is the use of fully humanized

monoclonal antibodies as described in U.S. Pat. No. 6,150,584 and respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 11

<210> SEQ ID NO 1

<211> LENGTH: 3534

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atgtggcgct ggatccggca gcagctgggt ttgacccac cacatcagag tgacacaaga      60
accatctacg tagccaacag gtttcctcag aatggccttt acacacctca gaaatttata      120
gataacagga tcatttcacg taagtacact gtgtggaatt ttgttccaaa aaattttatt      180
gaacagtcca gaagagtggc aaacttttat ttctctatta ttttttgggt tcagcttatg      240
attgatacac ctaccagtcg agttaccagt ggacttccat tattctttgt gataacagta      300
actgccataa agcagggata tgaagattgg ttacggcata actcagataa tgaagtaaat      360
ggagctcctg tttatgttgt tcgaagtggg ggccttgtaa aaactagatc aaaaaacatt      420
cgggtggggt atattgttcg aatagccaaa gatgaaattt ttcctgcaga cttggtgctt      480
ctgtcctcag atcgactgga tggttcctgt cacgttacaa ctgctagttt ggacggagaa      540
actaacctga agacacatgt ggcagttcca gaaacagcat tattacaac agttgccaat      600
ttggacactc tagtagctgt aatagaatgc cagcaaccag aagcagactt atacagattc      660
atgggacgaa tgatcataac ccaacaaatg gaagaaattg taagacctct ggggccggag      720
agtctcctcg ttcgtggagc cagattaaaa aacacaaaag aaatttttgg tgttgccgta      780
tacactgaaa tggaaactaa gatggcatta aattacaaga gcaaatcaca gaaacgatct      840
gcagtagaaa agtcaatgaa tacatttttg ataatttato tagtaattct tatactgaa      900
gctgtcatca gcactatctt gaagtataca tggcaagctg aagaaaaatg ggatgaacct      960
tggtataacc aaaaaacaga acatcaaaga aatagcagta agattctgag atttatttca      1020
gacttccttg cttttttggg tctctacaat ttcattcatc caatttcatt atatgtgaca      1080
gtcgaatatc agaaatttct tggatcattt tttattggct gggatcttga tctgtatcat      1140

```

-continued

gaagaatcag atcagaaagc tcaagtcaat acttccgac tgaatgaaga gcttggacag	1200
gtagagtacg tgtttacaga taaaactggg acactgacag aaaatgagat gcagtttcgg	1260
gaatgttcaa ttaatggcat gaaataccaa gaaattaatg gtagacttgt acccgaagga	1320
ccaacaccag actcttcaga aggaaactta tcttatctta gtagtttacc ccatcttaac	1380
aacttatccc atcttacaac cagttcctct ttcagaacca gtcctgaaaa tgaactgaa	1440
ctaattaaag aacatgatct cttctttaa gacgtcagtc tctgtcacac tgtacagatt	1500
agcaatgttc aaactgactg cactggtgat ggtccctggc aatccaacct ggcaccatcg	1560
cagttggagt actatgcac ttcaccagat gaaaaggctc tagtagaagc tgctgcaagg	1620
attggtattg tgttattgg caattctgaa gaaactatgg aggttaaaac tcttgaaaa	1680
ctggaacggt aaaaactgct tcatattctg gaatttgatt cagatcgtag gagaatgagt	1740
gtaattgttc aggcaccttc aggtgagaag ttattatttg cttaaaggagc tgagtcacca	1800
attctcccta aatgtatagg tggagaaata gaaaaacca gaattcatgt agatgaattt	1860
gcttgaaag ggctaagaac tctgtgtata gcatataga aatttacatc aaagagtat	1920
gaggaaatag ataaacgcat atttgaagcc aggactgcct tgcagcagcg ggaagagaaa	1980
ttggcagctg tttccagtt catagagaaa gacctgatat tacttgagc caccagcagta	2040
gaagacagac tacaagataa agttcgagaa actattgaag cattgagaat ggctggatc	2100
aaagtatggg tacttactgg ggataaacat gaaacagctg ttagtgtgag tttatcatgt	2160
ggccattttc atagaacat gaacatcctt gaaattataa accagaaatc agacagcgag	2220
tgtgtgaaac aattgaggca gcttgccaga agaattacag aggatcatgt gattcagcat	2280
gggctgtagt tggatgggac cagcctatct cttgcaacta gggagcatga aaaactattt	2340
atggaagttt gcagaaattg ttcagctgta ttatgtgtc gtatggctcc actgcagaaa	2400
gcaaaagtaa taagactaat aaaaatatca cctgagaaac ctataacatt ggtgttggt	2460
gatgtgtgta atgacgtaag catgatacaa gaagcccatg ttggcatagg aatcatgggt	2520
aaagaaggaa gacagcgtgc aagaacagc gactatgcaa tagccagatt taagtctctc	2580
tccaaattgc tttttgttca tggctatttt tattatatta gaatagctac cctgtacag	2640
tatttttttt ataagaatgt gtgctttatc acacccagc ttttatatca gttctactgt	2700
ttgttttctc agcaaacatt gtatgacagc gtgtacctga cttatataa tatttgtttt	2760
acttccctac ctattctgat atatagtctt ttggaacagc atgtagacc ccatgtgtta	2820
caaaataagc ccacccttta tcgagacatt agtaaaaacc gcctcttaag tattaataa	2880
tttctttatt ggaccatcct gggcttcagt catgccttta tttcttttt tggatcctat	2940
ttactaatag ggaagatag atctctgctt ggaatggcc agatgttygg aaactggaca	3000
tttggcactt tggctcttcc agtcatgggt attacagta cagtaagat ggctctggaa	3060
actcattttt ggacttggat caaccatctc gttacctggg gatctattat attttatttt	3120
gtattttcct tgttttatgg agggattctc tggccatttt tgggctccca gaatatgtat	3180
tttgtgttta ttcagctcct gtcaagtggg tctgcttggg ttgccataat cctcatgggt	3240
gttacatgtc tatttcttga tatcataaag aaggtctttg accgacacct ccaccctaca	3300
agtactgaaa aggcacagct tactgaaaca aatgcaggta tcaagtgtt ggactccatg	3360
tgctgtttcc cggaaggaga agcagcgtgt gcatctgttg gaagaatgct ggaacgagtt	3420
ataggaagat gtagtccaac ccacatcagc agatcatgga gtgcatcgga tctttctat	3480
accaacgaca ggagcatctt gactctctcc acaatggact catctacttg ttaa	3534

-continued

<210> SEQ ID NO 2
 <211> LENGTH: 1177
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

```

Met Trp Arg Trp Ile Arg Gln Gln Leu Gly Phe Asp Pro Pro His Gln
 1           5           10           15

Ser Asp Thr Arg Thr Ile Tyr Val Ala Asn Arg Phe Pro Gln Asn Gly
 20           25           30

Leu Tyr Thr Pro Gln Lys Phe Ile Asp Asn Arg Ile Ile Ser Ser Lys
 35           40           45

Tyr Thr Val Trp Asn Phe Val Pro Lys Asn Leu Phe Glu Gln Phe Arg
 50           55           60

Arg Val Ala Asn Phe Tyr Phe Leu Ile Ile Phe Leu Val Gln Leu Met
 65           70           75           80

Ile Asp Thr Pro Thr Ser Pro Val Thr Ser Gly Leu Pro Leu Phe Phe
 85           90           95

Val Ile Thr Val Thr Ala Ile Lys Gln Gly Tyr Glu Asp Trp Leu Arg
100           105           110

His Asn Ser Asp Asn Glu Val Asn Gly Ala Pro Val Tyr Val Val Arg
115           120           125

Ser Gly Gly Leu Val Lys Thr Arg Ser Lys Asn Ile Arg Val Gly Asp
130           135           140

Ile Val Arg Ile Ala Lys Asp Glu Ile Phe Pro Ala Asp Leu Val Leu
145           150           155           160

Leu Ser Ser Asp Arg Leu Asp Gly Ser Cys His Val Thr Thr Ala Ser
165           170           175

Leu Asp Gly Glu Thr Asn Leu Lys Thr His Val Ala Val Pro Glu Thr
180           185           190

Ala Leu Leu Gln Thr Val Ala Asn Leu Asp Thr Leu Val Ala Val Ile
195           200           205

Glu Cys Gln Gln Pro Glu Ala Asp Leu Tyr Arg Phe Met Gly Arg Met
210           215           220

Ile Ile Thr Gln Gln Met Glu Glu Ile Val Arg Pro Leu Gly Pro Glu
225           230           235           240

Ser Leu Leu Leu Arg Gly Ala Arg Leu Lys Asn Thr Lys Glu Ile Phe
245           250           255

Gly Val Ala Val Tyr Thr Gly Met Glu Thr Lys Met Ala Leu Asn Tyr
260           265           270

Lys Ser Lys Ser Gln Lys Arg Ser Ala Val Glu Lys Ser Met Asn Thr
275           280           285

Phe Leu Ile Ile Tyr Leu Val Ile Leu Ile Ser Glu Ala Val Ile Ser
290           295           300

Thr Ile Leu Lys Tyr Thr Trp Gln Ala Glu Glu Lys Trp Asp Glu Pro
305           310           315           320

Trp Tyr Asn Gln Lys Thr Glu His Gln Arg Asn Ser Ser Lys Ile Leu
325           330           335

Arg Phe Ile Ser Asp Phe Leu Ala Phe Leu Val Leu Tyr Asn Phe Ile
340           345           350

Ile Pro Ile Ser Leu Tyr Val Thr Val Glu Met Gln Lys Phe Leu Gly
355           360           365

Ser Phe Phe Ile Gly Trp Asp Leu Asp Leu Tyr His Glu Glu Ser Asp

```

-continued

370	375	380
Gln Lys Ala Gln Val Asn Thr Ser Asp Leu Asn Glu Glu Leu Gly Gln 385 390 395 400		
Val Glu Tyr Val Phe Thr Asp Lys Thr Gly Thr Leu Thr Glu Asn Glu 405 410 415		
Met Gln Phe Arg Glu Cys Ser Ile Asn Gly Met Lys Tyr Gln Glu Ile 420 425 430		
Asn Gly Arg Leu Val Pro Glu Gly Pro Thr Pro Asp Ser Ser Glu Gly 435 440 445		
Asn Leu Ser Tyr Leu Ser Ser Leu Ser His Leu Asn Asn Leu Ser His 450 455 460		
Leu Thr Thr Ser Ser Ser Phe Arg Thr Ser Pro Glu Asn Glu Thr Glu 465 470 475 480		
Leu Ile Lys Glu His Asp Leu Phe Phe Lys Ala Val Ser Leu Cys His 485 490 495		
Thr Val Gln Ile Ser Asn Val Gln Thr Asp Cys Thr Gly Asp Gly Pro 500 505 510		
Trp Gln Ser Asn Leu Ala Pro Ser Gln Leu Glu Tyr Tyr Ala Ser Ser 515 520 525		
Pro Asp Glu Lys Ala Leu Val Glu Ala Ala Ala Arg Ile Gly Ile Val 530 535 540		
Phe Ile Gly Asn Ser Glu Glu Thr Met Glu Val Lys Thr Leu Gly Lys 545 550 555 560		
Leu Glu Arg Tyr Lys Leu Leu His Ile Leu Glu Phe Asp Ser Asp Arg 565 570 575		
Arg Arg Met Ser Val Ile Val Gln Ala Pro Ser Gly Glu Lys Leu Leu 580 585 590		
Phe Ala Lys Gly Ala Glu Ser Ser Ile Leu Pro Lys Cys Ile Gly Gly 595 600 605		
Glu Ile Glu Lys Thr Arg Ile His Val Asp Glu Phe Ala Leu Lys Gly 610 615 620		
Leu Arg Thr Leu Cys Ile Ala Tyr Arg Lys Phe Thr Ser Lys Glu Tyr 625 630 635 640		
Glu Glu Ile Asp Lys Arg Ile Phe Glu Ala Arg Thr Ala Leu Gln Gln 645 650 655		
Arg Glu Glu Lys Leu Ala Ala Val Phe Gln Phe Ile Glu Lys Asp Leu 660 665 670		
Ile Leu Leu Gly Ala Thr Ala Val Glu Asp Arg Leu Gln Asp Lys Val 675 680 685		
Arg Glu Thr Ile Glu Ala Leu Arg Met Ala Gly Ile Lys Val Trp Val 690 695 700		
Leu Thr Gly Asp Lys His Glu Thr Ala Val Ser Val Ser Leu Ser Cys 705 710 715 720		
Gly His Phe His Arg Thr Met Asn Ile Leu Glu Leu Ile Asn Gln Lys 725 730 735		
Ser Asp Ser Glu Cys Ala Glu Gln Leu Arg Gln Leu Ala Arg Arg Ile 740 745 750		
Thr Glu Asp His Val Ile Gln His Gly Leu Val Val Asp Gly Thr Ser 755 760 765		
Leu Ser Leu Ala Leu Arg Glu His Glu Lys Leu Phe Met Glu Val Cys 770 775 780		
Arg Asn Cys Ser Ala Val Leu Cys Cys Arg Met Ala Pro Leu Gln Lys 785 790 795 800		

-continued

Ala Lys Val Ile Arg Leu Ile Lys Ile Ser Pro Glu Lys Pro Ile Thr
 805 810 815
 Leu Ala Val Gly Asp Gly Ala Asn Asp Val Ser Met Ile Gln Glu Ala
 820 825 830
 His Val Gly Ile Gly Ile Met Gly Lys Glu Gly Arg Gln Ala Ala Arg
 835 840 845
 Asn Ser Asp Tyr Ala Ile Ala Arg Phe Lys Phe Leu Ser Lys Leu Leu
 850 855 860
 Phe Val His Gly His Phe Tyr Tyr Ile Arg Ile Ala Thr Leu Val Gln
 865 870 875 880
 Tyr Phe Phe Tyr Lys Asn Val Cys Phe Ile Thr Pro Gln Phe Leu Tyr
 885 890 895
 Gln Phe Tyr Cys Leu Phe Ser Gln Gln Thr Leu Tyr Asp Ser Val Tyr
 900 905 910
 Leu Thr Leu Tyr Asn Ile Cys Phe Thr Ser Leu Pro Ile Leu Ile Tyr
 915 920 925
 Ser Leu Leu Glu Gln His Val Asp Pro His Val Leu Gln Asn Lys Pro
 930 935 940
 Thr Leu Tyr Arg Asp Ile Ser Lys Asn Arg Leu Leu Ser Ile Lys Thr
 945 950 955 960
 Phe Leu Tyr Trp Thr Ile Leu Gly Phe Ser His Ala Phe Ile Phe Phe
 965 970 975
 Phe Gly Ser Tyr Leu Leu Ile Gly Lys Asp Thr Ser Leu Leu Gly Asn
 980 985 990
 Gly Gln Met Phe Gly Asn Trp Thr Phe Gly Thr Leu Val Phe Thr Val
 995 1000 1005
 Met Val Ile Thr Val Thr Val Lys Met Ala Leu Glu Thr His Phe Trp
 1010 1015 1020
 Thr Trp Ile Asn His Leu Val Thr Trp Gly Ser Ile Ile Phe Tyr Phe
 1025 1030 1035 1040
 Val Phe Ser Leu Phe Tyr Gly Gly Ile Leu Trp Pro Phe Leu Gly Ser
 1045 1050 1055
 Gln Asn Met Tyr Phe Val Phe Ile Gln Leu Leu Ser Ser Gly Ser Ala
 1060 1065 1070
 Trp Phe Ala Ile Ile Leu Met Val Val Thr Cys Leu Phe Leu Asp Ile
 1075 1080 1085
 Ile Lys Lys Val Phe Asp Arg His Leu His Pro Thr Ser Thr Glu Lys
 1090 1095 1100
 Ala Gln Leu Thr Glu Thr Asn Ala Gly Ile Lys Cys Leu Asp Ser Met
 1105 1110 1115 1120
 Cys Cys Phe Pro Glu Gly Glu Ala Ala Cys Ala Ser Val Gly Arg Met
 1125 1130 1135
 Leu Glu Arg Val Ile Gly Arg Cys Ser Pro Thr His Ile Ser Arg Ser
 1140 1145 1150
 Trp Ser Ala Ser Asp Pro Phe Tyr Thr Asn Asp Arg Ser Ile Leu Thr
 1155 1160 1165
 Leu Ser Thr Met Asp Ser Ser Thr Cys
 1170 1175

<210> SEQ ID NO 3

<211> LENGTH: 1125

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 3

```

atgtggcgct ggatccggca gcagctgggt ttgaccac caccacagag tgacacaaga    60
accatctacg tagccaacag gtttctcag aatggccttt acacacctca gaaatttata    120
gataacagga tcatttcac taagtacact gtgtggaatt ttgtccaaa aaatttattt    180
gaacagttca gaagagtggc aaacttttat ttctttatta ttttttgggt tcagcttatg    240
attgatacac ctaccagtcc agttaccagt ggacttccat tattctttgt gataacagta    300
actgccataa agcagggata tgaagattgg ttacggcata actcagataa tgaagtaaat    360
ggagctcctg tttatgttg tcgaagtggg ggcttgtaa aaactagatc aaaaaacatt    420
cgggtgggtg atattgttcg aatagccaaa gatgaaattt ttctgcaga ctgtgtgctt    480
ctgtctcag atcgactgga tggttcctgt caggttaca ctgctagttt ggacggagaa    540
actaacctga agacacatgt ggcagttcca gaaacagcat tattacaac agttgccaat    600
ttggacactc tagtagctgt aatagaatgc cagcaaccag aagcagactt atacagattc    660
atgggacgaa tgatcataac ccaacaaatg gaagaaattg taagacctct ggggccggag    720
agtctcctgc tctgtggagc cagattaaaa aacacaaaag aaatttttgg tgttgccgta    780
tacactggaa tggaaactaa gatggcatta aattacaaga gcaaatcaca gaaacgatct    840
gcagtagaaa agtcaatgaa tacatttttg ataatttct tagtaattct tatatctgaa    900
gctgtcatca gcactatctt gaagtataca tggcaagctg aagaaaaatg ggatgaacct    960
tggтатаacc aaaaaacaga acatcaaaaga aatagcaatt ctgagattta ttccagactt   1020
ccttgctttt ttggttctct acaatttcat cattccaatt tcattatatg tgacagtcga   1080
aatgcagaaa tttcttggat cattttttat tggctgggat cttga                      1125

```

<210> SEQ ID NO 4

<211> LENGTH: 374

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 4

```

Met Trp Arg Trp Ile Arg Gln Gln Leu Gly Phe Asp Pro Pro His Gln
 1             5             10             15
Ser Asp Thr Arg Thr Ile Tyr Val Ala Asn Arg Phe Pro Gln Asn Gly
      20             25             30
Leu Tyr Thr Pro Gln Lys Phe Ile Asp Asn Arg Ile Ile Ser Ser Lys
      35             40             45
Tyr Thr Val Trp Asn Phe Val Pro Lys Asn Leu Phe Glu Gln Phe Arg
      50             55             60
Arg Val Ala Asn Phe Tyr Phe Leu Ile Ile Phe Leu Val Gln Leu Met
65             70             75             80
Ile Asp Thr Pro Thr Ser Pro Val Thr Ser Gly Leu Pro Leu Phe Phe
      85             90             95
Val Ile Thr Val Thr Ala Ile Lys Gln Gly Tyr Glu Asp Trp Leu Arg
      100            105            110
His Asn Ser Asp Asn Glu Val Asn Gly Ala Pro Val Tyr Val Val Arg
      115            120            125
Ser Gly Gly Leu Val Lys Thr Arg Ser Lys Asn Ile Arg Val Gly Asp
      130            135            140
Ile Val Arg Ile Ala Lys Asp Glu Ile Phe Pro Ala Asp Leu Val Leu
      145            150            155            160
Leu Ser Ser Asp Arg Leu Asp Gly Ser Cys His Val Thr Thr Ala Ser

```

-continued

165	170	175
Leu Asp Gly Glu Thr Asn Leu Lys Thr His Val Ala Val Pro Glu Thr 180 185 190		
Ala Leu Leu Gln Thr Val Ala Asn Leu Asp Thr Leu Val Ala Val Ile 195 200 205		
Glu Cys Gln Gln Pro Glu Ala Asp Leu Tyr Arg Phe Met Gly Arg Met 210 215 220		
Ile Ile Thr Gln Gln Met Glu Glu Ile Val Arg Pro Leu Gly Pro Glu 225 230 235 240		
Ser Leu Leu Leu Arg Gly Ala Arg Leu Lys Asn Thr Lys Glu Ile Phe 245 250 255		
Gly Val Ala Val Tyr Thr Gly Met Glu Thr Lys Met Ala Leu Asn Tyr 260 265 270		
Lys Ser Lys Ser Gln Lys Arg Ser Ala Val Glu Lys Ser Met Asn Thr 275 280 285		
Phe Leu Ile Ile Tyr Leu Val Ile Leu Ile Ser Glu Ala Val Ile Ser 290 295 300		
Thr Ile Leu Lys Tyr Thr Trp Gln Ala Glu Glu Lys Trp Asp Glu Pro 305 310 315 320		
Trp Tyr Asn Gln Lys Thr Glu His Gln Arg Asn Ser Asn Ser Glu Ile 325 330 335		
Tyr Phe Arg Leu Pro Cys Phe Phe Gly Ser Leu Gln Phe His His Ser 340 345 350		
Asn Phe Ile Ile Cys Asp Ser Arg Asn Ala Glu Ile Ser Trp Ile Ile 355 360 365		
Phe Tyr Trp Leu Gly Ser 370		

<210> SEQ ID NO 5

<211> LENGTH: 7277

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 5

```

gccgcgggat gggaacgcgg cgcggggagt gaggcagtgg cggcggcggc ggtaagcggg      60
acttcggccc gaggggctcg cccgcctccc cctctgtctt gtcggcctcc acctgcagcc      120
ccgcggcccc cgcgccccgc gggacccgga cggcgacgac gggggaatgt ggcgctggat      180
ccggcagcag ctgggttttg acccaccaca tcagagtgc acaagaacca tctacgtagc      240
caacagggtt cctcagaatg gcctttacac acctcagaaa tttatagata acaggatcat      300
ttcatctaag tacactgtgt ggaattttgt tccaaaaaat ttatttgaac agttcagaag      360
agtggcaaac ttttatttcc ttattatatt tttggttcag cttatgattg atacacctac      420
cagtccagtt accagtggac ttccattatt ctttgtgata acagtaactg ccataaagca      480
gggatatgaa gattgggttac ggcataaactc agataatgaa gtaaatggag ctctgttcta      540
tgttgttcga agtgggtggc ttgtaaaaac tagatcaaaa aacattcggg tgggtgatata      600
tgttcgaata gccaaagatg aaatttttcc tgcagacttg gtgcttctgt cctcagatcg      660
actggatggg tcctgtcacg ttacaactgc tagtttggac ggagaaacta acotgaagac      720
acatgtggca gttccagaaa cagcattatt acaaacagtt gccaatggg acactctagt      780
agctgtaata gaatgccagc aaccagaagc agacttatac agattcatgg gacgaatgat      840
cataacccaa caaatggaag aaattgtaag acctctgggg ccggagagtc tcctgcttcg      900

```

-continued

tgagccaga ttaaaaaa caaaagaat ttttggtgtt gcggtataca ctggaatgga	960
aactaagatg gcattaaatt acaagagcaa atcacagaaa cgatctgcag tagaaaagtc	1020
aatgaatata tttttgataa tttatctagt aattcttata tctgaagctg tcatcagcac	1080
tatcttgaag tatacatggc aagctgaaga aaaatgggat gaaccttggt ataaccaaaa	1140
aacagaacat caaagaata gcagtaagat tctgagattt atttcagact tccttgcttt	1200
tttggtcttc tacaatttca tcattccaat ttcattatat gtgacagtcg aaatgcagaa	1260
atttcttgga tcatttttta ttggctggga tcttgatctg tatcatgaag aatcagatca	1320
gaaagctcaa gtcaataact ccgatctgaa tgaagagctt ggacaggtag agtacgtgtt	1380
tacagataaa actggtacac tgacagaaaa tgagatgcag tttcgggaat gttcaattaa	1440
tggcatgaaa taccagaata ttaatggtag acttgtacct gaaggaccaa caccagactc	1500
ttcagaagga aacttatctt atcttagtag tttatcccat cttacaact tatccatct	1560
tacaaccagt tctcttttca gaaccagtcc tgaataatgaa actgaactaa ttaagaaca	1620
tgatctcttc tttaaagcag tcagtctctg tcacactgta cagattagca atgttcaaac	1680
tgactgcact ggtgatggtc cctggcaatc caacctggca ccacgcagat tggagtacta	1740
tgcatcttca ccagatgaaa aggccttagt agaagctgct gcaaggattg gtattgtgtt	1800
tattggcaat tctgaagaaa ctatggaggt taaaactctt ggaaaactgg aacggtacaa	1860
actgcttcat attctggaat ttgattcaga tcgtaggaga atgagtgtaa ttgttcaggc	1920
accttcaggt gagaagtat tatttgctaa aggagctgag tcatcaattc tccctaaatg	1980
tataggtgga gaaatagaaa aaaccagaat tcatgtagat gaatttgctt tgaagggtct	2040
aagaactctg tgtatagcat atagaaaatt tacatcaaaa gagtatgagg aaatagataa	2100
acgcataatt gaagccagga ctgccttgca gcagcgggaa gagaaattgg cagctgtttt	2160
ccagttcata gagaaagacc tgatattact tggagccaca gcagtagaag acagactaca	2220
agataaagtt cgagaaacta ttgaagcatt gagaatggct ggtatcaag tatgggtact	2280
tactggggat aaacatgaaa cagctgttag tgtgagtta tcatgtggc attttcatag	2340
aacctgaac atccttgaa ttataaacca gaaatcagac agcgagtgtg ctgaacaatt	2400
gaggcagctt gccagaagaa ttacagagga tcatgtgatt cagcatgggc tggtagtgga	2460
tgggaccagc ctatctcttg cactcaggga gcataaaaa ctatttatgg aagtttgag	2520
aaattgttca gctgtattat gctgtcgtat ggctccactg cagaaagcaa aagtaataag	2580
actaataaaa atatcacctg agaaacctat aacattggct gttggtgatg gtgctaatga	2640
cgtaagcatg atacaagaag cccatgttgg cataggaaac atgggtaaaag aaggaagaca	2700
ggctgcaaga aacagtgact atgcaatagc cagatttaag ttcctctcca aattgctttt	2760
tgttcatggt catttttatt atattagaat agctaccctt gtacagtatt tttttataa	2820
gaatgtgtgc tttatcacac cccagttttt atatcagttc tactgtttgt tttctcagca	2880
aacattgtat gacagcgtgt acctgacttt atacaatatt tgttttactt ccctacctat	2940
tctgatatat agtcttttgg aacagcatgt agaccctcat gtgttacaaa ataagccac	3000
cctttatcga gacattagta aaaaccgcct cttaagtatt aaacatttc tttattggac	3060
catcctgggc ttcagtcagc cctttatttt cttttttgga tcctatttac taatagggaa	3120
agatacatct ctgcttgtaa atggccagat gtyygaaac tggacatttg gcactttggt	3180
cttcacagtc atggttatta cagtcacagt aaagatggct ctggaaactc atttttggac	3240
tggatcaac catctcgtta cctggggatc tatttatatt tttttgtat tttcctgtt	3300

-continued

ttagggagg	attctctggc	catttttggg	ctcccagaat	atgtattttg	tgttatttca	3360
gctcctgtca	agtgggtctg	cttggtttgc	cataatcctc	atgggtgtta	catgtctatt	3420
tcttgatata	ataaagaagg	tctttgaccg	acacctccac	cctacaagta	ctgaaaaggc	3480
acagcttact	gaaacaaatg	caggtatcaa	gtgcttggac	tccatgtgct	gtttcccgga	3540
aggagaagca	gcgtgtgcat	ctgttgaag	aatgctggaa	cgagttatag	gaagatgtag	3600
tccaacccac	atcagcagat	catggagtgc	atcggtacct	ttctatacca	acgacaggag	3660
catcttgact	ctctccacaa	tggactcatc	tacttgtaa	aggggcagta	gtactttgtg	3720
ggagccagtt	cacctccttt	cctaaaattc	agtgtgatca	ccctgttaat	ggccacacta	3780
gctctgaat	taatttccaa	aatctttgta	gtagttcata	cccactcaga	gttataatgg	3840
caaacaaaca	gaaagcatta	gtacaagccc	ctcccaaac	ccttaatttg	aactctgaaca	3900
tgtaaaatt	tgagaataaa	gagacatttt	tcattctctt	gtctggtttg	tcccttgtgc	3960
ttaggggact	cctaattgga	tttcagtctg	ttgctgaggc	cattatattt	taataataat	4020
gtagaaaaaa	gagagaaatc	ttagtaaaaga	gtatttttta	gtattagctt	gattattgac	4080
tcttctattt	aaatctgctt	ctgtaaatia	tgctgaaagt	ttgccttgag	aactctattt	4140
ttttattaga	gttatattta	aagcttttca	tgggaaaagt	taatgtgaat	actgaggaat	4200
tttggtccct	cagtgcacctg	tggtgttaat	tcattaatgc	attctgagtt	cacagagcaa	4260
attaggagaa	tcatttccaa	ccattattta	ctgcagtatg	gggagtaaat	ttataccaat	4320
tcctctaact	gtactgtaac	acagcctgta	aagtttagcca	tataaatgca	agggtatatc	4380
atatatacaa	atcaggaatc	aggtccgttc	accgaacttc	aaattgatgt	ttactaatat	4440
ttttgtgaca	gagtataaag	accctatagt	gggtaaatta	gatactatta	gcataattatt	4500
aatttaaatgt	ctttatcatt	ggatcttttg	catgctttta	tctgggttaac	atattttaat	4560
ttgctttttt	tctctttacc	tgaaggctct	gtgtatagta	tttcatgaca	tcgtttgtaca	4620
gtttaactat	atcaataaaa	agtttgga	gtattttaat	attgcaaata	tgtttaatta	4680
tacaaatcag	aatagtagtg	gtaattaaat	gaatacaaaa	agaagagcct	ctttctgcag	4740
ccgacttaga	catgctcttc	cctttctata	agctagattt	tagaataaag	ggtttcagtt	4800
aataatctta	ttttcaggtt	atgtcatcta	acttatagca	aactaccaca	atcacgtgag	4860
ttctgccagt	gtcccagtac	aaggcatatt	tcagggtgtg	ctgtggaatg	taaaaatgct	4920
caacttgat	caggtaatgt	tagcaataaa	ttaaatgcta	agaatgatta	atcgggtaca	4980
tgttactgta	attaactcat	tgcaattcaa	aacctaactt	ccatcctgaa	tttatcaagt	5040
agttcagtat	tgctatttgt	ttttgtttta	ttgaaaagta	atgttgtctt	aagattttaga	5100
agtgattatt	agcttgagaa	ctattaccca	gctctaagca	aataatgatt	gtatacatat	5160
taagataatg	gttaaatgcy	gttttaccaa	gttttccctt	gaaaatgtaa	ttcctttatg	5220
gagatttatt	gtgcagccct	aagcttcctt	cccatttcat	gaatataagg	cttctagaat	5280
tggactggca	ggggaaagaa	tggtagagac	agaaattaag	actttatcct	tggttgcttg	5340
taaaactatta	ttttcttgct	aatgtaacat	ttgtctgttc	cagtgatgta	aggatattaa	5400
gttattaagc	taaatattaa	ttttcaaaaa	tagtccttct	ttaacttaga	tatttcatag	5460
ctggatttag	gaagatctgt	tattctggaa	gtactaaaaa	gaataataca	acgtacaatg	5520
tctgcattca	ctaattcatg	ttccagaaga	ggaaataatg	aagatatact	cagtagagta	5580
ctaggtggga	ggatatggaa	atttgctcat	aaaatctctt	ataaaacgtg	catataacaa	5640

-continued

aatgacacc	agtaggcctg	cattacattt	acatgaccgt	gtttatttgc	catcaataa	5700
actgagtact	gacaccagac	aaagactcca	aagtcataaa	atagcctatg	accaactgca	5760
gcaagacagg	aggtcagctc	gcctataatg	gtgcttaaag	tgtgattgat	gtaattttct	5820
gtactcacca	tttgaagtta	gttaaggaga	actttatttt	tttaaaaaaa	gtaaattggca	5880
accactagt	tgctcactct	gaactgttac	tccaaatcca	ctccgttttt	aaagcaaat	5940
tatcttgtga	ttttaagaaa	agagttttct	atattattaa	gaaagtaaca	atgcagtctg	6000
caagctttca	gtagttttct	agtgcctat	tcactcctga	aaactcttac	tacgtaacca	6060
gtaatcacaa	ggaaagtgtc	ccctttgcat	atctctttta	aattctttct	ttggaagtta	6120
tgatgttgat	aattaactta	cccttatctg	ccaaaaccag	agcaaatgc	taaatcgtt	6180
attgctaate	agtgggtctca	aatcgatttg	cctccctttg	cctcgtctga	gggctgtaag	6240
cctgaagata	gtggcaagca	ccaagtcagt	ttccaaaatt	gcccctcagc	tgctttaagt	6300
gactcagcac	cctgcctcag	cttcagcagg	cstaggctca	ccctgggcgg	agcaaatgat	6360
gggccaggga	gaactacagc	tacgaagacc	tgctgtcag	ttgagaaaag	gggagaattt	6420
atggtctgaa	ttttctaact	gtcctctttc	ttgggtctaa	agtcataat	acacaaaggc	6480
ttccagacct	gagccacacc	caggccctat	cctgaacagg	agactaaaca	gaggcaaatc	6540
aaccttagga	aatacttgca	ttctgcccta	cgttagtagc	caggactgag	gtcattttcta	6600
ctggaaaaga	ttgtgagatt	gaacttatct	gacgccttga	gactccta	aggcaggagt	6660
caaggccact	agaaaattga	cagttaagag	ccaaaagttt	ttaaaatatg	ctactctgaa	6720
aaatctcgtg	aaggctgtag	gaaaaggag	aatcttccat	gttggtgttt	ttcctgtaaa	6780
gatcagtttg	gggtatgata	taagcaggtta	tttaaaaaaa	taacacacca	aagagttacg	6840
taaaacatgt	tttattaatt	ttggtcccca	cgtacagaca	ttttatttct	attttgaaat	6900
gagttatcta	ttttcataaa	agtaaaacac	tattaaagtg	ctgttttatg	tgaataaact	6960
tgaatgttgt	tcctataaaa	aatagatcat	aactcatgat	atgtttgtaa	tcattgtaat	7020
ttagattttt	atgaggaatg	agtatctgga	aattattgtg	caataacttg	tttaaaattt	7080
tggacctgag	acactgtggc	tgtctaattg	aatcctttta	aaattctctg	cattgtcagt	7140
aatgtagta	tattattgtg	cagctactca	taatttttta	aagtttatga	agttatattt	7200
atcaataaaa	aactttccta	tataattaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa	7260
aaaaaaaaaa	aaaaaaaaaa					7277

<210> SEQ ID NO 6

<211> LENGTH: 2913

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 6

atggcacac	tagagaggag	cgccatctct	ggcttcagct	ctaagtcag	gcgaaactca	60
ttcgcatatg	atgttaagcg	tgaagtatac	aatgaggaga	cctttcaaca	ggaacacaaa	120
aggaaggcct	cctcttcttg	gaacatgaac	atcaacatca	ccaccttcag	acaccacgtc	180
cagtgcgct	gctcatggca	caggttccta	cgatgertgc	ttacaatctt	tccttctcta	240
gaatggatgt	gtatgtatcg	attaaaggat	tggttcttg	gagacttact	tgctgggata	300
agtgttggcc	ttgtgcaagt	tccccaaggc	ctgacactta	gtttgctggc	aaggcaactg	360
attctctctc	tcaacatcgc	ttatgcagct	ttctgttctt	cggtaatcta	tgtaattttt	420
ggatcgtgtc	atcaaatgtc	cgttggttcc	ttcttctctg	tgagtgtctt	gctgatcaac	480

-continued

gtttgaaag tgagccatt caacaacggt caactgggtca tgggatcttt cgtcaagaat	540
gagttttcgg cccctccta ccttatgggc tataataaat ccttgagtgt ggtggcaacc	600
acaacttttc tgactgggat tattcagcta ataattggcg tattgggttt gggcttcatt	660
gccacttacc ttccggagtc tgcaatgaat gcttacctgg ctgctgtggc acttcataac	720
atgctgtccc agctgacttt catctttggg attatgatta gtttccatgc cgtgccatc	780
tccttcttct atgacataat taattactgt gtagctctcc caaaagcgaa ttccaccagc	840
attctagtat ttctaactgt tgttgttgc ctgcgaatca acaaatgtat cagaatttct	900
ttcaatcagt atccattga gtttcccatg gaattatttc tgattattgg cttcactgtg	960
attgcaaac agataagcat ggccacagaa accagccaga cgcttattga catgattcct	1020
tatagcttcc tgcttctgt aacaccagat ttcagccttc ttccaagat aattttacaa	1080
gccttctct tctctttggt gagctccttt ctgctcatat ttctgggcaa gaagattgcc	1140
agtcttcaca attacagtgt caattccaac caggatttaa tagccatcgg cctttgcaat	1200
gtcgtcagtt cttttttcag atcttgtgtg tttactgggt ctattgctag gactattatc	1260
caggataaat ctggaggaa acaacagttt gcctctctgg taggcgcagg tgtgatgctg	1320
ctcctgatgg tgaagatggg acacttttcc tacacactgc caaatgctgt gctggctggt	1380
attattctga gcaacgtcat tcctacctt gaaaccattt ctaacctacc cagcctgtgg	1440
aggcaggacc aatatgactg tgctctttgg atgatgacat tctcatcttc aattttcctg	1500
ggactggaca ttggactaat tatctcagta gtttctgctt tcttcatac cactgttcgt	1560
tcacacagag ctaagattct tctcctgggt caaatcccta acaccaacat ttatagaagc	1620
atcaatgatt atcgggagat catcaccatt cctgggggtga aaatcttcca gtgctgcagc	1680
tcaattacat ttgtaaatgt ttactaccta aagcataagc tgtaaaaga ggttgatatg	1740
gtaaagggtc ctcttaaaga agaagaaatt ttcagcttgt ttaattcaag tgacaccaat	1800
ctacaaggag gaaagatttg cagggttttc tgcaactgtg atgatctgga gccgctgccc	1860
aggattcttt acacagagcg atttgaaaat aaactggatc ccgaagcatc ctccattaac	1920
ctgattcact gctcacattt tgagagcatg aacacaagcc aaactgcac cgaagaccaa	1980
gtgccataca cagtatcgtc cgtgtctcag aaaaatcaag ggcaacagta tgaggagggtg	2040
gaggaaagtt ggcttcctaa taactcatca agaaacagct caccaggact gcctgatgtg	2100
gcggaaagcc aggggaggag atcactcatc cttactcag atgcgtctct actgccagat	2160
gtccacacca tcactctgga tttctccatg gtacactacg tggattcacg ggggttagtc	2220
gtattaagac agatattgaa tgccctttcaa aacgccaaca ttttgatact cattgcaggg	2280
tgtcactctt ccatagtcag ggcatttgag aggaatgatt tctttgacgc tggcatcacc	2340
aagaccacgc tgttctcag cgttcacgac gccgtgctgt ttgccttgtc aaggaaggtc	2400
ataggctcct ctgagttaaag catcgatgaa tccgagacag tgatacggga aacctactca	2460
gaaacagaca agaattgaca ttcaagatat aaaatgagca gcagttttct aggaagccaa	2520
aaaaatgtaa gtccaggctt catcaagatc caacagcctg tagaagagga gtcggagttg	2580
gatttgagc tggaatcaga acaagaggct gggctgggtc tggacctaga cctggatcgg	2640
gagctggagc ctgaaatgga gcccaaggct gagaccgaga ccaagaccca gaccgagatg	2700
gagccccagc ctgagactga gcctgagatg gagcccaacc ccaaatctag gccaaagact	2760
cacacttttc ctacagagcg ttactggcct atgtatcatc cgtctatggc ttccaccag	2820

-continued

tctcagactc agactcggac atgggtcagt gagaggagac gccatcctat ggattcatac 2880

tcaccagagg gcaacagcaa tgaagatgtc tag 2913

<210> SEQ ID NO 7
 <211> LENGTH: 970
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: VARIANT
 <222> LOCATION: (1)...(970)
 <223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 7

Met Ala Gln Leu Glu Arg Ser Ala Ile Ser Gly Phe Ser Ser Lys Ser
 1 5 10 15
 Arg Arg Asn Ser Phe Ala Tyr Asp Val Lys Arg Glu Val Tyr Asn Glu
 20 25 30
 Glu Thr Phe Gln Gln Glu His Lys Arg Lys Ala Ser Ser Ser Gly Asn
 35 40 45
 Met Asn Ile Asn Ile Thr Thr Phe Arg His His Val Gln Cys Arg Cys
 50 55 60
 Ser Trp His Arg Phe Leu Arg Cys Met Leu Thr Ile Phe Pro Phe Leu
 65 70 75 80
 Glu Trp Met Cys Met Tyr Arg Leu Lys Asp Trp Leu Leu Gly Asp Leu
 85 90 95
 Leu Ala Gly Ile Ser Val Gly Leu Val Gln Val Pro Gln Gly Leu Thr
 100 105 110
 Leu Ser Leu Leu Ala Arg Gln Leu Ile Pro Pro Leu Asn Ile Ala Tyr
 115 120 125
 Ala Ala Phe Cys Ser Ser Val Ile Tyr Val Ile Phe Gly Ser Cys His
 130 135 140
 Gln Met Ser Val Gly Ser Phe Phe Leu Val Ser Ala Leu Leu Ile Asn
 145 150 155 160
 Val Leu Lys Val Ser Pro Phe Asn Asn Gly Gln Leu Val Met Gly Ser
 165 170 175
 Phe Val Lys Asn Glu Phe Ser Ala Pro Ser Tyr Leu Met Gly Tyr Asn
 180 185 190
 Lys Ser Leu Ser Val Val Ala Thr Thr Thr Phe Leu Thr Gly Ile Ile
 195 200 205
 Gln Leu Ile Met Gly Val Leu Gly Leu Gly Phe Ile Ala Thr Tyr Leu
 210 215 220
 Pro Glu Ser Ala Met Asn Ala Tyr Leu Ala Ala Val Ala Leu His Ile
 225 230 235 240
 Met Leu Ser Gln Leu Thr Phe Ile Phe Gly Ile Met Ile Ser Phe His
 245 250 255
 Ala Gly Pro Ile Ser Phe Phe Tyr Asp Ile Ile Asn Tyr Cys Val Ala
 260 265 270
 Leu Pro Lys Ala Asn Ser Thr Ser Ile Leu Val Phe Leu Thr Val Val
 275 280 285
 Val Ala Leu Arg Ile Asn Lys Cys Ile Arg Ile Ser Phe Asn Gln Tyr
 290 295 300
 Pro Ile Glu Phe Pro Met Glu Leu Phe Leu Ile Ile Gly Phe Thr Val
 305 310 315 320
 Ile Ala Asn Lys Ile Ser Met Ala Thr Glu Thr Ser Gln Thr Leu Ile
 325 330 335

-continued

Asp Met Ile	Pro Tyr Ser	Phe Leu Leu	Pro Val Thr	Pro Asp Phe Ser
340		345		350
Leu Leu Pro	Lys Ile Ile	Leu Gln Ala	Phe Ser Leu	Ser Leu Val Ser
355		360		365
Ser Phe Leu	Leu Ile Phe	Leu Gly Lys	Lys Ile Ala	Ser Leu His Asn
370		375		380
Tyr Ser Val	Asn Ser Asn	Gln Asp Leu	Ile Ala Ile	Gly Leu Cys Asn
385		390		395
Val Val Ser	Ser Phe Phe	Arg Ser Cys	Val Phe Thr	Gly Ala Ile Ala
	405		410	415
Arg Thr Ile	Ile Gln Asp	Lys Ser Gly	Gly Arg Gln	Gln Phe Ala Ser
	420		425	430
Leu Val Gly	Ala Gly Val	Met Leu Leu	Met Val Lys	Met Gly His
	435		440	445
Phe Phe Tyr	Thr Leu Pro	Asn Ala Val	Leu Ala Gly	Ile Ile Leu Ser
	450		455	460
Asn Val Ile	Pro Tyr Leu	Glu Thr Ile	Ser Asn Leu	Pro Ser Leu Trp
	465		470	475
Arg Gln Asp	Gln Tyr Asp	Cys Ala Leu	Trp Met Met	Thr Phe Ser Ser
	485		490	495
Ser Ile Phe	Leu Gly Leu	Asp Ile Gly	Leu Ile Ile	Ser Val Val Ser
	500		505	510
Ala Phe Phe	Ile Thr Thr	Val Arg Ser	His Arg Ala	Lys Ile Leu Leu
	515		520	525
Leu Gly Gln	Ile Pro Asn	Thr Asn Ile	Tyr Arg Ser	Ile Asn Asp Tyr
	530		535	540
Arg Glu Ile	Ile Thr Ile	Pro Gly Val	Lys Ile Phe	Gln Cys Cys Ser
	545		550	555
Ser Ile Thr	Phe Val Asn	Val Tyr Tyr	Leu Lys His	Lys Leu Leu Lys
	565		570	575
Glu Val Asp	Met Val Lys	Val Pro Leu	Lys Glu Glu	Glu Ile Phe Ser
	580		585	590
Leu Phe Asn	Ser Ser Asp	Thr Asn Leu	Gln Gly Gly	Lys Ile Cys Arg
	595		600	605
Cys Phe Cys	Asn Cys Asp	Asp Leu Glu	Pro Leu Pro	Arg Ile Leu Tyr
	610		615	620
Thr Glu Arg	Phe Glu Asn	Lys Leu Asp	Pro Glu Ala	Ser Ser Ile Asn
	625		630	635
Leu Ile His	Cys Ser His	Phe Glu Ser	Met Asn Thr	Ser Gln Thr Ala
	645		650	655
Ser Glu Asp	Gln Val Pro	Tyr Thr Val	Ser Ser Val	Ser Gln Lys Asn
	660		665	670
Gln Gly Gln	Gln Tyr Glu	Glu Val Glu	Glu Val Trp	Leu Pro Asn Asn
	675		680	685
Ser Ser Arg	Asn Ser Ser	Pro Gly Leu	Pro Asp Val	Ala Glu Ser Gln
	690		695	700
Gly Arg Arg	Ser Leu Ile	Pro Tyr Ser	Asp Ala Ser	Leu Leu Pro Ser
	705		710	715
Val His Thr	Ile Ile Leu	Asp Phe Ser	Met Val His	Tyr Val Asp Ser
	725		730	735
Arg Gly Leu	Val Val Leu	Arg Gln Ile	Cys Asn Ala	Phe Gln Asn Ala
	740		745	750
Asn Ile Leu	Ile Leu Ile	Ala Gly Cys	His Ser Ser	Ile Val Arg Ala
	755		760	765

-continued

Phe Glu Arg Asn Asp Phe Phe Asp Ala Gly Ile Thr Lys Thr Gln Leu
 770 775 780
 Phe Leu Ser Val His Asp Ala Val Leu Phe Ala Leu Ser Arg Lys Val
 785 790 795 800
 Ile Gly Ser Ser Glu Leu Ser Ile Asp Glu Ser Glu Thr Val Ile Arg
 805 810 815
 Glu Thr Tyr Ser Glu Thr Asp Lys Asn Asp Asn Ser Arg Tyr Lys Met
 820 825 830
 Ser Ser Ser Phe Leu Gly Ser Gln Lys Asn Val Ser Pro Gly Phe Ile
 835 840 845
 Lys Ile Gln Gln Pro Val Glu Glu Glu Ser Glu Leu Asp Leu Glu Leu
 850 855 860
 Glu Ser Glu Gln Glu Ala Gly Leu Gly Leu Asp Leu Asp Leu Asp Arg
 865 870 875 880
 Glu Leu Glu Pro Glu Met Glu Pro Lys Ala Glu Thr Glu Thr Lys Thr
 885 890 895
 Gln Thr Glu Met Glu Pro Gln Pro Glu Thr Glu Pro Glu Met Glu Pro
 900 905 910
 Asn Pro Lys Ser Arg Pro Arg Ala His Thr Phe Pro Gln Gln Arg Tyr
 915 920 925
 Trp Pro Met Tyr His Pro Ser Met Ala Ser Thr Gln Ser Gln Thr Gln
 930 935 940
 Thr Arg Thr Trp Ser Val Glu Arg Arg Arg His Pro Met Asp Ser Tyr
 945 950 955 960
 Ser Pro Glu Gly Asn Ser Asn Glu Asp Val
 965 970

<210> SEQ ID NO 8
 <211> LENGTH: 3749
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 8

ttttccaact ccccatctcc tccctcctca gattaaaaga agttatatgg actttgtgat 60
 gttttctgcc gctttgtgaa gtaggcctta tttctcttgt ctttcgtac agggaggaat 120
 ttgaagtaga tagaaaccga cctggattac tccggtctga actcagatca cgtaggactt 180
 taatcgttga acaaacgaac cttaaatagc ggctgcacca tcgggatgtc ctgatccaac 240
 atcgaggtcg taaaccttat tgttgatatg gactctagaa taggattgag ctgttatccc 300
 tagggtaact tgttccgttg gtcaagttat tgatcaatt gactatagta gttcgtttg 360
 actggtgaag tcttggtatg tactgctcgg aggttgggtt ctgctccgag gtcgccccaa 420
 ccgaaatttt taatgcagga gcgccgcac tcccgcccc gccaaaggag caggaatggc 480
 acaactagag aggagcgcca tctctggctt cagctctaag tccaggcgaa actcattcgc 540
 atatgatgtt aagcgtgaag tataaatga ggagacctt caacaggaac acaaaaggaa 600
 ggctctctct tctgggaaca tgaacatcaa catcaccacc ttcagacacc acgtccagt 660
 ccgctgctca tggcacaggt tctacgatg crtgttaca atctttccct tctagaatg 720
 gatgtgtatg tatcgattaa aggattggct tctggggagac ttacttctg gtataagtgt 780
 tggccttctg caagtcccc aaggcctgac acttagtttg ctggcaaggc aactgattcc 840
 tctctcaac atcgcttatg cagctttctg ttcttcgcta atctatgtaa tttttggatc 900
 gtgtcatcaa atgtccgttg gttccttctt cctggtgagt gctctgctga tcaacgttct 960

-continued

gaaagtgagc ccattcaaca acggtcaact ggtcatggga tctttcgtca agaagtgatt	1020
ttcggccccc tctacctta tgggtataa taaatccttg agtggtggg caaccacaac	1080
ttttctgact gggattatc agctaataat gggcgtattg ggtttgggct tcattgccac	1140
ttacctccg gagtctgcaa tgaatgctta cctggctgct gtggcacttc atatcatgct	1200
gtcccagctg actttcatct ttgggattat gattagtctc catgcggctc ccatctcctt	1260
cttctatgac ataattaatt actgtgtagc tctcccaaaa gcgaattcca ccagcattct	1320
agtatttcta actgtgttg ttgctctcg aatcaacaan tgtatcagaa tttctttcaa	1380
tcagtatccc attgagtttc ccatggaatt atttctgatt attggcttca ctgtgattgc	1440
aaacaagata agcatggcca cagaaccag ccagacgctt attgacatga ttccttatag	1500
ctttctgctt cctgtaacac cagatttcag ccttcttccc aagataattt tacaagcctt	1560
ctccttatct ttggtgagct cctttctgct catatttctg ggcaagaaga ttgccagtct	1620
tcacaattac agtgtcaatt ccaaccagga tttaatagcc atcggccttt gcaatgtcgt	1680
cagttcattt ttcagatctt gtgtgtttac tgggtctatt gctaggacta ttatccagga	1740
taaactctga ggaagacaac agtttgcatc tctggtaggc gcaggtgtga tgctgctcct	1800
gatggtgaa atggggacact tttctacac actgccaaat gctgtgctgg ctggtattat	1860
tctgagcaac gtcattccct accttgaac catttctaac ctaccagcc tgtggaggca	1920
ggaccaatat gactgtgctc ttggatgat gacattctca tcttcaattt tcctgggact	1980
ggacattgga ctaattatct cagtagtttc tgctttcttc atcaccactg ttcgttcaca	2040
cagagctaag attcttctcc tgggtcaaat ccctaacacc aacatttata gaagcatcaa	2100
tgattatcgg gagatcatca ccattcctgg ggtgaaaac tccagtgct gcagctcaat	2160
tacatttgta aatgtttact acctaagca taagctgtta aaagaggttg atatggtaaa	2220
ggtgcctctt aaagaagaag aaattttcag cttgtttaat tcaagtga ccaatctaca	2280
aggaggaaag atttgaggt gtttctgcaa ctgtgatgat ctggagccgc tgcccaggat	2340
tctttacaca gagcgatttg aaaaataact ggatcccgaa gcacccctca ttaacctgat	2400
tcaactgtca cattttgaga gcatgaacac aagccaaact gcacccgaag accaagtgcc	2460
atacacagta tcgtccgtgt ctcagaaaaa tcaagggcaa cagtatgagg aggtggagga	2520
agtttggtt cctaataact catcaagaaa cagctcacca ggactgcctg atgtggcgga	2580
aagccagggg aggagatcac tcatccctta ctcagatgct tctctactgc ccagtgtcca	2640
caccatcacc ctggatttct ccatggtaca ctacgtggat tcacgggggt tagtctgatt	2700
aagacagata tgcaatgcct ttcaaaacgc caacattttg atactcattg caggggtgca	2760
ctcttcata gtcagggcat ttgagaggaa tgatttcttt gacgctggca tcaccaagac	2820
ccagctgttc ctcagcgttc acgacgcctg gctgtttgcc ttgtcaagga aggtcatagg	2880
ctcctctgag ttaangcatg atgaatccga gacagtata cgggaaacct actcagaaac	2940
agacaagaat gacaattcaa gatataaat gagcagcagt tttctaggaa gccaaaaaaa	3000
tgtaagtcca ggcttcacat agatccaaca gcctgtagaa gaggagtcgg agttggattt	3060
ggagctggaa tcagaacaag aggctgggct gggctctggac ctagacctgg atcgggagct	3120
ggagcctgaa atggagccca aggtctgagc cgagaccaag acccagaccg agatggagcc	3180
ccagcctgag actgagcctg agatggagcc caaccccaaa tctaggccaa gagctcacac	3240
ttttcctcag cagcgttact ggcctatgta tcatccgtct atgggttcca cccagtctca	3300

-continued

gactcagact cggacatggt cagtggagag gagacgccat cctatggatt catactcacc	3360
agagggcaac agcaatgaag atgtctagga gatgaactag aaataagggg tcagataatg	3420
ctggcaaato ctctaccca aaaaggggtc aattgtccag agacctagac tggatacgaa	3480
ctagcagtac ttcttctctg actgtgactc ctactacctg ccagccttct tccttgcctc	3540
gcgctgggat catactccca aatcacatta cttaaatgcca acaattatct ctgaattccc	3600
tatccaggct cccctcattt caccttcagc atatatctta gtcataaatt tccttcttca	3660
cacacccccc atctctgggc ttgtgcccag accatctcta acttaactct ctcatccctg	3720
ttcccttttc tccaaagaga tgaagctca	3749

<210> SEQ ID NO 9
 <211> LENGTH: 1524
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 9

atgggggtgtt ggggtcggaa ccggggcccg ctgctgtgca tgctggcgct gaccttcattg	60
ttcatgtgtc tggaggtgtt ggtgagcccg gtgacctcgt cgctggcgat gctctccgac	120
tccttcaca tgctgtcggc cgtgctggcg ctggtgtgtg cgctgggtgc cgagcgcttc	180
gcccgccgga cccacgccac ccagaagaac acgttcggct ggatccgagc cgaggtaatg	240
ggggctctgg tgaacgccat ettcctgact ggctctgtt tcgccatcct gctggaggcc	300
atcgagcgct tcatcgagcc gcacgagatg cagcagccgc tgggtgtcct tggggtcggc	360
gtggccgggc tgctgtgtcaa cgtgctgggg ctctgcctct tcacccatca cagcggttc	420
agccaggact ccggccacgg ccactcgcac ggggggtcac gccacggcca cggcctcccc	480
aaggggcctc gcgttaagag caccgcctcc gggagcagcg acatcaacgt ggccccgggc	540
gagcagggtc ccgaccagga ggagaccaac accctggtgg ccaataccag caactccaac	600
gggtgaaat tggacccccc agaccagaa aacccagaa gtggtgatac agtggaagta	660
caagtgaatg gaaatcttgt cagagaacct gaccatatgg aactggaaga agatagggct	720
ggacaactta acatgcgtgg agttttcttg catgtccttg gagatgcctt gggttcagt	780
attgtatag taaatgcctt agtcttttac tttcttga aaggtgttc tgaaggggat	840
ttttgtgta atccatgttt ccctgacccc tgcaagcat ttgtagaat aattaatagt	900
actcatgcat cactttatga ggctgttcct tgctgggtgc tataattaga tccaactctt	960
tgtgttgtaa tggtttgtat acttctttac acaacctatc cattaactaa ggaatctgct	1020
cttattcttc tacaaactgt tcctaaacaa attgatatca gaaatttgat aaaagaactt	1080
cgaaatgttg aaggagtga ggaagttcat gaattacatg tttggcaact tgctggaagc	1140
agaatcattg ccactgctca cataaaatgt gaagatccaa catcatacat ggaggtggct	1200
aaaaccatta aagacgtttt tcataatcac ggaattcacg ctactaccat tcagcctgaa	1260
tttgctagt taggtcttaa atcaagtga gtccgtgtg aacttgctg cagaacccag	1320
tgtgcttga agcaatgttg tgggacacta ccacaagccc cttatggaaa ggtgcagaa	1380
aagacccagc cagttagcat ttctgttta gaacttagta acaatctaga gaagaagccc	1440
aggaggacta aagctgaaaa catccctgct gttgtgtag agattaataa catgccaaac	1500
aaacaacctg aatcatcttt gtga	1524

<210> SEQ ID NO 10
 <211> LENGTH: 507

-continued

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 10

```

Met Gly Cys Trp Gly Arg Asn Arg Gly Arg Leu Leu Cys Met Leu Ala
 1           5           10           15

Leu Thr Phe Met Phe Met Val Leu Glu Val Val Val Ser Arg Val Thr
 20           25           30

Ser Ser Leu Ala Met Leu Ser Asp Ser Phe His Met Leu Ser Asp Val
 35           40           45

Leu Ala Leu Val Val Ala Leu Val Ala Glu Arg Phe Ala Arg Arg Thr
 50           55           60

His Ala Thr Gln Lys Asn Thr Phe Gly Trp Ile Arg Ala Glu Val Met
 65           70           75           80

Gly Ala Leu Val Asn Ala Ile Phe Leu Thr Gly Leu Cys Phe Ala Ile
 85           90           95

Leu Leu Glu Ala Ile Glu Arg Phe Ile Glu Pro His Glu Met Gln Gln
100           105           110

Pro Leu Val Val Leu Gly Val Gly Val Ala Gly Leu Leu Val Asn Val
115           120           125

Leu Gly Leu Cys Leu Phe His His His Ser Gly Phe Ser Gln Asp Ser
130           135           140

Gly His Gly His Ser His Gly Gly His Gly His Gly His Gly Leu Pro
145           150           155           160

Lys Gly Pro Arg Val Lys Ser Thr Arg Pro Gly Ser Ser Asp Ile Asn
165           170           175

Val Ala Pro Gly Glu Gln Gly Pro Asp Gln Glu Glu Thr Asn Thr Leu
180           185           190

Val Ala Asn Thr Ser Asn Ser Asn Gly Leu Lys Leu Asp Pro Ala Asp
195           200           205

Pro Glu Asn Pro Arg Ser Gly Asp Thr Val Glu Val Gln Val Asn Gly
210           215           220

Asn Leu Val Arg Glu Pro Asp His Met Glu Leu Glu Glu Asp Arg Ala
225           230           235           240

Gly Gln Leu Asn Met Arg Gly Val Phe Leu His Val Leu Gly Asp Ala
245           250           255

Leu Gly Ser Val Ile Val Val Val Asn Ala Leu Val Phe Tyr Phe Ser
260           265           270

Trp Lys Gly Cys Ser Glu Gly Asp Phe Cys Val Asn Pro Cys Phe Pro
275           280           285

Asp Pro Cys Lys Ala Phe Val Glu Ile Ile Asn Ser Thr His Ala Ser
290           295           300

Leu Tyr Glu Ala Gly Pro Cys Trp Val Leu Tyr Leu Asp Pro Thr Leu
305           310           315           320

Cys Val Val Met Val Cys Ile Leu Leu Tyr Thr Thr Tyr Pro Leu Leu
325           330           335

Lys Glu Ser Ala Leu Ile Leu Leu Gln Thr Val Pro Lys Gln Ile Asp
340           345           350

Ile Arg Asn Leu Ile Lys Glu Leu Arg Asn Val Glu Gly Val Glu Glu
355           360           365

Val His Glu Leu His Val Trp Gln Leu Ala Gly Ser Arg Ile Ile Ala
370           375           380

Thr Ala His Ile Lys Cys Glu Asp Pro Thr Ser Tyr Met Glu Val Ala
385           390           395           400

```

-continued

Lys Thr Ile Lys Asp Val Phe His Asn His Gly Ile His Ala Thr Thr
 405 410 415
 Ile Gln Pro Glu Phe Ala Ser Val Gly Ser Lys Ser Ser Val Val Pro
 420 425 430
 Cys Glu Leu Ala Cys Arg Thr Gln Cys Ala Leu Lys Gln Cys Cys Gly
 435 440 445
 Thr Leu Pro Gln Ala Pro Tyr Gly Lys Asp Ala Glu Lys Thr Pro Ala
 450 455 460
 Val Ser Ile Ser Cys Leu Glu Leu Ser Asn Asn Leu Glu Lys Lys Pro
 465 470 475 480
 Arg Arg Thr Lys Ala Glu Asn Ile Pro Ala Val Val Ile Glu Ile Lys
 485 490 495
 Asn Met Pro Asn Lys Gln Pro Glu Ser Ser Leu
 500 505

<210> SEQ ID NO 11
 <211> LENGTH: 2222
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 11

ctccggctgc ggcctcttgg accccggctc cgggagccca gctccccgcc accgcccgcg 60
 cctgggtgtg ggggctgctg aggctgagcc gggcttcggc gccggctctg aggacggacg 120
 cctgaggagc tgcgcggcgc ggcgccgcgc gctggcggag aacgcccaca ggcgcggggc 180
 tcggcggtt gaccggggtt tgctcccgct cggccggggg ggccctcag cggtttcccg 240
 aacggccccg ctgcggcgct cctccgtgtc gcggtcgccg accctccgcg tcccgccaac 300
 gccgcgctg caccagtctc cgggcccggc tcggcgggcc ccgcagccgc agccatgggg 360
 tgttggggg ggaaccgggg ccggctgctg tgcctgctgg cgctgacctt catgttcatg 420
 gtgctggagg tgggtgtgag ccgggtgacc tcgtcgctgg cgatgctctc cgactccttc 480
 cacatgctgt cggacgtgct ggcgctggtg gtggcgctgg tggccgagcg cttcgcccg 540
 cggacccacg ccaccagaa gaacacgttc ggctggatcc gagccgaggt aatgggggct 600
 ctgggtgaac ccattcttct gactggcctc tgtttcgcca tctgctgga ggccatcgag 660
 cgcttcacg agccgcacga gatgcagcag ccgctggtgg tccttggggg cggcggtggc 720
 gggctgctgg tcaactgctt ggggctctgc ctctccacc atcacagcg cttcagccag 780
 gactccggcc acggccactc gcacgggggt caccggccag gccacggcct ccccaagggg 840
 cctcgcggtt agagcaccgc ccccgggagc agcgacatca acgtggcccc gggcgagcag 900
 ggtcccagac aggaggagac caacacccct gtggccaata ccagcaactc caacgggctg 960
 aaattggacc ccgcagacct agaaaacccc agaagtgggt atacagtgga agtacaagt 1020
 aatggaaatc ttgtcagaga acctgacct atggaactgg aagaagatag ggctggacaa 1080
 cttaacatgc gtggagtttt tctgcatgtc cttggagatg ctttgggttc agtgattgta 1140
 gtagtaaatg ccttagtctt ttacttttct tggaaagggt gttctgaagg ggattttgt 1200
 gtgaatccat gtttccctga ccctgcaaa gcattttagt aaataattaa tagtactcat 1260
 gcatacttt atgaggctgg tccttgcctg gtgctatatt tagatccaac tctttgtgtt 1320
 gtaatgggtt gtatacttct ttacacaacc tatccattac ttaaggaatc tgctcttatt 1380
 cttctacaaa ctgttcttaa acaaatgat atcagaaatt tgataaaga acttcgaaat 1440
 gttgaaggag ttgaggaagt tcatgaatta catgtttggc aacttgcctg aagcagaatc 1500

-continued

attgccactg ctcacataaa atgtgaagat ccaacatcat acatggaggt ggctaaaacc	1560
attaaagacg tttttcataa tcacggaatt cacgctacta ccattcagcc tgaatttgc	1620
agtgtaggct ctaaatacaag tgtagtccg tgtgaacttg cctgcagaac ccagtgtgt	1680
ttgaagcaat gttgtgggac actaccacaa gcccttatg gaaaggatgc agaaaagacc	1740
ccagcagtta gcatttcttg tttagaactt agtaacaatc tagagaagaa gcccaggagg	1800
actaaagctg aaaacatccc tgctgttggt atagagatta aaaacatgcc aaacaacaa	1860
cctgaatcat ctttgtgagt cttgaaaaag atgtgatatt tgacttttgc tttaaactgc	1920
aagaggaaaa agactccact gaaattctaa gtttgccaag tagtgtaatt gaagtccttg	1980
tctggtcaca cagtttaatt ctatttttgc aagaacataa tgggactgca taacagagtt	2040
ctatattaca atttgtgatt attagtacag agtacagcta tgctgtgact gttttggaaa	2100
gccagtttta acactatggt acatttttgc ttaaagtaag ttaaacctta tataacataa	2160
tgacatttga tttctggatt tttcccatgg ataaaaaatt aggggggata aaattaaat	2220
tg	2222

What is claimed is:

1. An isolated nucleic acid molecule comprising a sequence that:

- (a) encodes the amino acid sequence shown in SEQ ID NO: 7; and
- (b) hybridizes under highly stringent conditions with wash conditions of 0.1×SSC/0.1%SDS at 68° C. to the nucleotide sequence of SEQ ID NO: 6 or the complement thereof.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 7.

3. A recombinant expression vector comprising the isolated nucleic acid molecule of claim 2.

4. A host cell comprising the recombinant expression vector of claim 3.

* * * * *

Q

This Page Blank (uspto)



US006511840B1

(12) **United States Patent**
Turner, Jr. et al.

(10) Patent No.: **US 6,511,840 B1**
(45) Date of Patent: **Jan. 28, 2003**

(54) **HUMAN KINASE PROTEINS AND
POLYNUCLEOTIDES ENCODING THE
SAME**

(75) Inventors: **C. Alexander Turner, Jr., The
Woodlands, TX (US); Brian Mathur,
The Woodlands, TX (US); Daniel
Mathur, Wooster, OH (US); Carl
Johan Friddle, The Woodlands, TX
(US)**

(73) Assignee: **Lexicon Genetics Incorporated, The
Woodlands, TX (US)**

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/883,134**

(22) Filed: **Jun. 15, 2001**

Related U.S. Application Data

(60) Provisional application No. 60/211,572, filed on Jun. 15,
2000, and provisional application No. 60/216,382, filed on
Jul. 7, 2000.

(51) Int. Cl.⁷ **C12N 1/20; C12N 15/00;
C12N 9/12; C07H 21/04; C07H 21/02**

(52) U.S. Cl. **435/252.3; 435/320.1;
435/6; 435/194; 536/23.1; 536/23.2**

(58) Field of Search **536/23.2, 23.1;
435/6, 320.1, 252.3, 194**

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,215,051 A	7/1980	Schroeder et al.
4,376,110 A	3/1983	David et al.
4,594,595 A	6/1986	Struckman
4,631,211 A	12/1986	Houghten
4,689,405 A	8/1987	Frank et al.
4,713,326 A	12/1987	Dattagupta et al.
4,873,191 A	10/1989	Wagner et al.
4,946,778 A	8/1990	Ladner et al.
5,252,743 A	10/1993	Barrett et al.
5,424,186 A	6/1995	Fodor et al.
5,445,934 A	8/1995	Fodor et al.
5,459,127 A	10/1995	Felgner et al.
5,556,752 A	9/1996	Lockhart et al.
5,700,637 A	12/1997	Southern
5,744,305 A	4/1998	Fodor et al.
5,830,721 A	11/1998	Stemmer et al.
5,837,458 A	11/1998	Minshull et al.
5,869,336 A	2/1999	Meyer et al.
5,877,397 A	3/1999	Lonberg et al.
5,948,767 A	9/1999	Scheule et al.
6,001,593 A	12/1999	Bandman et al.
6,075,181 A	6/2000	Kucherlapati et al.
6,110,490 A	8/2000	Thierry
6,150,584 A	11/2000	Kucherlapati et al.

OTHER PUBLICATIONS

EST Database, Avccession No. BE736116, Sep. 2000.*
Hillier et al., EST Database, Accession No. AA088547, Jul.
1997.*

Bird et al, 1988, "Single-Chain Antigen-Binding Proteins",
Science 242:423-426.

Bitter et al, 1987, "Expression and Secretion Vectors for
Yeast", Methods in Enzymology 153:516-544.

Colbere-Garapin et al, 1981, "A New Dominant Hybrid
Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol.
150:1-14.

Gautier et al, 1987, "α-DNA IV: α-anomeric and β-anomeric
tetrathymidylates covalently linked to intercalating
oxazolopyridocarbazole. Synthesis, physicochemical properties
and poly (rA) binding", Nucleic Acids Research
15(16):6625-6641.

Gordon, 1989, "Transgenic Animals", International Review
of Cytology, 115:171-229.

Greenspan et al, 1993, "Idiotypes: structure and immuno-
genicity", FASEB Journal 7:437-444.

Gu et al, 1994, "Deletion of a DNA Polymerase β Gene
Segment in T Cells Using Cell Type-Specific Gene Target-
ing", Science 265:103-106.

Huse et al, 1989, "Generation of a Large Combinatorial
Library of the Immunoglobulin Repertoire in Phage
Lambda", Science 246:1275-1281.

Huston et al, 1988, "Protein engineering of antibody binding
sites: Recovery of specific activity in an anti-digoxin single-
chain Fv analogue produced in *Escherichia coli*", Proc.
Natl. Acad. Sci. USA 85:5879-5883.

Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA
using modified oligonucleotides splints and R Nase H",
FEBS Letters 215(2):327-330.

Inoue et al, 1987, "Synthesis and hybridization studies on
two complementary nona(2'-O-methyl)ribonucleotides",
Nucleic Acids Research 15(15):6131-6149.

Inouye & Inouye, 1985, "Up-promoter mutations in the lpp
gene of *Escherichia coli*", Nucleic Acids Research
13(9):3101-3110.

Janknecht et al, 1991, "Rapid and efficient purification of
native histidine-tagged protein expressed by recombinant
vaccinia virus", PNAS 88:8972-8976.

Kohler & Milstein, 1975, "Continuous cultures of fused
cells secreting antibody of predefined specificity", Nature
256:495-497.

Lakso et al, 1992, "Targeted oncogene activation by
site-specific recombination in transgenic mice", Proc. Natl.
Acad. Sci. USA 89:6232-6236.

Lavitrano et al, 1989, "Sperm Cells ad Vectors for Intro-
ducing Foreign DNA into Eggs: Genetic Transformation of
Mice", Cell 57:717-723.

Lo, 1983, "Transformation by Iontophoretic Microinjection
of DNA: Multiple Integrations without Tandem Insertions",
Mol. & Cell. Biology 3(10):1803-1814.

(List continued on next page.)

Primary Examiner—Rebecca E. Prouty
Assistant Examiner—Maryam Monshipouri

(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are
disclosed that can be used in therapeutic, diagnostic, and
pharmacogenomic applications.

5 Claims, No Drawings

OTHER PUBLICATIONS

- Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection", Proc. Natl. Acad. Sci. USA 81:3655-3659.
- Lowy et al, 1980, "Isolation of Transforming DNA: Cloning the Hamster apt Gene", Cell 22:817-823.
- Morrison et al, 1984, "Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains", Proc. Natl. Acad. Sci. USA 81:6851-6855.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", Proc. Natl. Acad. Sci. USA 78(4):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies possessing novel effector functions," Nature 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", J. of Immunology 147:2429-2438.
- O'Hare et al, 1981, "Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase", Proc. Natl. Acad. Sci. USA 78(3):1527-1531.
- Ruther et al, 1983, "Easy identification of cDNA clones", EMBO Journal 2(10):1791-1794.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", Gene 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", Proc. Natl. Acad. Sci. USA 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", J. Virol. 46(2):584-593.
- Stein et al, 1988, "Physicochemical properties of phosphorothioate oligodeoxynucleotides", Nucleic Acids Research 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", Proc. Natl. Acad. Sci. USA 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", Nature 314:452-454.
- Thompson et al, 1989, "Germ Line Transmission and Expression of a Corrected HPRT Gene Produced by Gene Targeting in Embryonic Stem Cells", Cell 56:313-321.
- Van Der Putten et al, 1985, "Efficient insertion of genes into the mouse germ line via retroviral vectors", Proc. Natl. Acad. Sci. USA 82:6148-6152.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", J. Biol. Chemistry 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", Cell 11:223-232.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", Proc. Natl. Acad. Sci. USA 77(6):3567-3570.

* cited by examiner

HUMAN KINASE PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application Nos. 60/211,572 and 60/216,382 which were filed on Jun. 15, 2000 and Jul. 7, 2000, respectively. These U.S. Provisional Applications are herein incorporated by reference in their entirety.

INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding a protein that shares sequence similarity with animal kinases. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed polynucleotides, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed polynucleotides that can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of physiological disorders or diseases, and cosmetic or nutraceutical applications.

BACKGROUND OF THE INVENTION

Kinases mediate phosphorylation of a wide variety of proteins and compounds in the cell. Along with phosphatases, kinases are involved in a range of regulatory pathways. Given the physiological importance of kinases, they have been subject to intense scrutiny and are proven drug targets.

SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins, and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal kinases, including, but not limited to myosin kinases and unconventional myosin classes of proteins (SEQ ID NOS:1-5) as well as serine-threonine kinases, calcium/calmodulin-dependent kinases 10 and MAP kinases (SEQ ID NOS:6-11). As such, the novel polynucleotides encode a new kinase protein having homologues and orthologs across a range of phyla and species.

The novel human polynucleotides described herein, encode open reading frames (ORFs) encoding proteins of 238, 1,236, 974, 922 and 255 amino acids in length (see respectively SEQ ID NOS: 2, 4, 7, 9, 11).

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof, that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHP polynucleotides (e.g., expression constructs that place the described polynucleotide under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" (which can be conditional) that do not express a functional NHP. Knock-out mice can

be produced in several ways, one of which involves the use of mouse embryonic stem cells ("ES cells") lines that contain gene trap mutations in a murine homolog of at least one of the described NHPs. When the unique NHP sequences described in SEQ ID NOS:1-11 are "knocked-out" they provide a method of identifying phenotypic expression of the particular gene as well as a method of assigning function to previously unknown genes. Additionally, the unique NHP sequences described in SEQ ID NOS:1-11 are useful for the identification of coding sequence, the identification of the actual biologically relevant exon splice junctions and the mapping of a unique gene to a particular chromosome.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequence of the novel human ORFs encoding the described novel human kinase proteins. SEQ ID NO:5 describes a full length NHP ORF and flanking regions.

DETAILED DESCRIPTION OF THE INVENTION

The NHP, described for the first time herein, are novel proteins that are widely expressed. NHP SEQ ID NO:1-5 are expressed in, inter alia, human cell lines, and human pituitary, lymph node, kidney, testis, thyroid, fetal kidney, and gene trapped cells. NHP SEQ ID NO:1-5 were compiled from gene trapped sequences in conjunction with sequences available in GENBANK, and cDNAs from a kidney mRNA (Edge Biosystems, Gaithersburg, Md.).

The NHPs, described for the first time in NHP SEQ ID NO:6-11 are novel proteins expressed in, inter alia, human cell lines, and human pituitary, lymph node, kidney, colon, and prostate cells. HP SEQ ID NO:6-11 were compiled from sequences available in GENBANK, and cDNAs generated from kidney, prostate, and colon mRNA (Edge Biosystems, Gaithersburg, Md.).

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described polynucleotides, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of the NHPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal (or hydrophobic transmembrane) sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of an NHP, or one of its domains (e.g., a receptor or ligand binding domain, accessory protein/

self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of a DNA sequence that encodes and expresses an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, *supra*), yet still encodes a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP nucleotide sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-11 can be used as a hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics,

polymers, metal or metallized substrates, crystalline or polycrystalline substrates, etc.). Of particular note are spatially addressable arrays (i.e., gene chips, microtiter plates, etc.) of oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-11, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, inter alia, U.S. Pat. Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-11 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-11.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap.

Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-11 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

Probes consisting of sequences first disclosed in SEQ ID NOS:1-11 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

As an example of utility, the sequences first disclosed in SEQ ID NOS:1-11 can be utilized in microarrays or other assay formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences

first disclosed in SEQ ID NOS:1-11 in silico and by comparing previously collected genetic databases and the disclosed sequences using computer software known to those in the art.

Thus the sequences first disclosed in SEQ ID NOS:1-11 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific oligonucleotide sequence(s) first disclosed in the SEQ ID NOS: 1-11. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (e.g., the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, Mich., etc.), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene.

The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be

labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, connective tissue disorders, infertility, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue can be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, N.Y.).

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a

result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869,336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of the NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor for an NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act

on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP receptor. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

The NHP Sequences

The cDNA sequences and corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing.

Expression analysis has provided evidence that the NHPs described in SEQ ID NO: 1-5 can be expressed in a relatively narrow range of human tissues. In addition to myosin III kinases, the NHPs described in SEQ ID NO: 1-5 also share significant similarity to a range of additional kinase families, including kinases associated with signal transduction, from a variety of phyla and species.

A number of polymorphisms can occur in the NHPs described in SEQ ID NO: 1-5, such as a possible A-G transition that can occur in the sequence region corresponding to, for example, nucleotide position 889 of SEQ ID NO: 3 that can result in a K or E being present in the corresponding amino acid sequence represented by, for example, position 297 of SEQ ID NO: 4. Similar myosin-like proteins, as well as uses and applications that are also applicable to the NHPs described in SEQ ID NO: 1-5, are described in U.S. Pat. No. 6,001,593 herein incorporated by reference in its entirety.

Expression analysis has provided evidence that the NHPs described in SEQ ID NO: 6-11 can be expressed in a relatively narrow range of human tissues. In addition to serine-threonine kinases, the NHPs described in SEQ ID NO: 6-11 also share significant similarity to a range of additional kinase families, again including kinases associated with signal transduction from a variety of phyla and species. The NHPs described in SEQ ID NO: 6-11 are apparently encoded on human chromosome 16.

An additional application of the described novel human polynucleotide sequences is their use in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Pat. Nos. 5,830,721 and 5,837,458 which are herein incorporated by reference in their entirety.

NHP gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate NHP transgenic animals.

Any technique known in the art may be used to introduce a NHP transgene into animals to produce the founder lines

of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the NHP transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al., 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that a NHP transgene be integrated into the chromosomal site of the endogenous NHP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NHP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NHP gene (i.e., "knockout" animals).

The transgene can also be selectively introduced into a particular cell type, thus inactivating the endogenous NHP gene in only that cell type, by following, for example, the teaching of Gu et al., 1994, Science, 265:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NHP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of NHP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the NHP transgene product.

NHPS and NHP Polypeptides

NHPs, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPS, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include but are not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to a NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and diseases. Given the similarity information and expression data, the described

NHPs can be targeted (by drugs, oligos, antibodies, etc.) in order to treat disease, or to therapeutically augment the efficacy of, for example, chemotherapeutic agents used in the treatment of breast or prostate cancer.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP polynucleotides. The NHPs typically display have initiator methionines in DNA sequence contexts consistent with a translation initiation site.

The NHP amino acid sequences of the invention include the amino acid sequence presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a substrate of a NHP, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where, as in the present instance, the NHP peptide or polypeptide is thought to be membrane protein, the hydrophobic regions of the protein can be excised and the resulting soluble peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, in situ. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host

cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Pharmacia or American Type Culture Collection) can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted sequence is expressed (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence.

This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phospho-

ribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp⁺ or apr⁺ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg⁺, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Also encompassed by the present invention are fusion proteins that direct the NHP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHPs to antibody molecules or their Fab fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHP would also transport the NHP to the desired location within the cell. Alternatively targeting of NHP or its nucleic acid sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in "Liposomes: A Practical Approach", New, RRC ed., Oxford University Press, New York and in U.S. Pat. Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective disclosures which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHP to the target site or desired organ. This goal may be achieved by coupling of the NHP to a cytokine or other ligand that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. applications Ser. Nos. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes if needed and can optionally be engineered to include nuclear localization sequences when desired.

Antibodies to NHP Products

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal

amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction with gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with a NHP, an NHP peptide (e.g., one corresponding to a functional domain of an NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger et al., 1984, *Nature*, 312:604-608; Takeda et al., 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of

appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Pat. Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety. Also encompassed by the present invention is the use of fully humanized monoclonal antibodies as described in U.S. Pat. No. 6,150,584 and respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, *Science* 242:423-426; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 341:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, *FASEB J* 7(5):437-444; and Nissinoff, 1991, *J. Immunol.* 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 11

<210> SEQ ID NO 1

<211> LENGTH: 717

<212> TYPE: DNA

-continued

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atgatgcttg gacttgaatc acttccagat cccacagaca cctgggaaat tatagagacc   60
attggtaaag gcacctatgg caaagtctac aaggtaacta acaagagaga tgggagcctg   120
gctgcagtga aaattctgga tccagtcagt gatatggatg aagaaattga ggcagaatac   180
aacattttgc agttccttcc taatcatccc aatgttgtaa agttttatgg gatgttttac   240
aaagcggatc actgtgtagg gggacagctg tggctgtgcc tggagctgtg taatgggggc   300
tcagtcacyg agcttgtcaa aggtctactc agatgtggcc agcggttgga tgaagcaatg   360
atctcataca tctgtacagg ggccctcttg ggccttcagc attgcacaa caaccgaatc   420
atccaccgtg atgtgaaggg gaataacatt cttctgacaa cagaaggagg agttaagctc   480
gttgactttg gtgtttcagc tcaactcacc agtacacgtc tgcggagaaa cacatctgtt   540
ggcaccccat tctggatggc cctgagggtc attgcctgtg agcagcagta tgactcttcc   600
tatgacgctc gctgtgacgt ctggtccttg gggatcacag ctattgaact gggggatgga   660
gacctccccc tctttgacat gcactcctgtg aaaacactct ttaagattcc aaggtaa   717

```

<210> SEQ ID NO 2

<211> LENGTH: 238

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

```

Met Met Leu Gly Leu Glu Ser Leu Pro Asp Pro Thr Asp Thr Trp Glu
 1      5      10      15
Ile Ile Glu Thr Ile Gly Lys Gly Thr Tyr Gly Lys Val Tyr Lys Val
 20      25      30
Thr Asn Lys Arg Asp Gly Ser Leu Ala Ala Val Lys Ile Leu Asp Pro
 35      40      45
Val Ser Asp Met Asp Glu Glu Ile Glu Ala Glu Tyr Asn Ile Leu Gln
 50      55      60
Phe Leu Pro Asn His Pro Asn Val Val Lys Phe Tyr Gly Met Phe Tyr
 65      70      75      80
Lys Ala Asp His Cys Val Gly Gly Gln Leu Trp Leu Val Leu Glu Leu
 85      90      95
Cys Asn Gly Gly Ser Val Thr Glu Leu Val Lys Gly Leu Leu Arg Cys
100      105      110
Gly Gln Arg Leu Asp Glu Ala Met Ile Ser Tyr Ile Leu Tyr Gly Ala
115      120      125
Leu Leu Gly Leu Gln His Leu His Asn Asn Arg Ile Ile His Arg Asp
130      135      140
Val Lys Gly Asn Asn Ile Leu Leu Thr Thr Glu Gly Gly Val Lys Leu
145      150      155      160
Val Asp Phe Gly Val Ser Ala Gln Leu Thr Ser Thr Arg Leu Arg Arg
165      170      175
Asn Thr Ser Val Gly Thr Pro Phe Trp Met Ala Pro Glu Val Ile Ala
180      185      190
Cys Glu Gln Gln Tyr Asp Ser Ser Tyr Asp Ala Arg Cys Asp Val Trp
195      200      205
Ser Leu Gly Ile Thr Ala Ile Glu Leu Gly Asp Gly Asp Pro Pro Leu
210      215      220
Phe Asp Met His Pro Val Lys Thr Leu Phe Lys Ile Pro Arg

```

-continued

225	230	235	
<210> SEQ ID NO 3			
<211> LENGTH: 3711			
<212> TYPE: DNA			
<213> ORGANISM: homo sapiens			
<400> SEQUENCE: 3			
atgatgcttg	gacttgaatc	acttccagat	cccacagaca cctgggaaat tatagagacc 60
attggtaaa	gcacctatgg	caaagcttac	aaggtaacta acaagagaga tgggagcctg 120
gctgcagtg	aaattctgga	tccagtcagt	gatatggatg aagaattga ggcagaatac 180
aacattttg	agttccttcc	taatcatccc	aatgttgtaa agttttatgg gatgttttac 240
aaagcggatc	actgtgtagg	gggacagctg	tggctgggtc tggagctgtg taatgggggc 300
tcagtcacyg	agcttgtcaa	aggtctactc	agatgtggcc agcggttgga tgaagcaatg 360
atctcataca	tcttgtacgg	ggccctcttg	ggccttcagc atttgacaa caaccgaatc 420
atccaccctg	atgtgaaggg	gaataacatt	cttctgacaa cagaaggagg agttaagctc 480
gttgactttg	gtgttttcag	tcaactcacc	agtacacgtc tgcggagaaa cacatctgtt 540
ggcaccceat	tctggatggc	ccctgaggtc	attgectgtg agcagcagta tgactcttcc 600
tatgacgctc	gctgtgacgt	ctggtccttg	gggatcacag ctattgaact gggggatgga 660
gaccctcccc	tctttgacat	gcacctctgt	aaaacactct ttaagattcc aagaatcct 720
ccacctactt	tacttcaccc	agaaaaatgg	tgtgaagaat tcaaccactt tatttcacag 780
tgctttatta	aggattttga	aaggcgacct	tccgtcacac atctccttga caccceatct 840
attaagggag	tacatggaaa	agttctgttt	ctgcaaaaac agctggccra ggttctccaa 900
gaccagaagc	atcaaaaatc	tggtgctaaa	accaggcatg agaggatgca taccagaaga 960
ccttatcatg	tggaaagatg	tgaaaaatcc	tgcccttgagg atgatttggt caacctagag 1020
gttctggatg	aggatacaat	tatccatcag	ttgcagaaac gttatgcaga cttgctaatt 1080
tacacatatg	ttggagacat	cttaattgcc	ttaaaccctc tccagaatct aagcatatac 1140
tctccacagt	ttccagact	ttatcatggg	gtgaaacgag cctccaaycc ccccccata 1200
tttgcacag	cagatgctgc	ttaccagtgc	atggttactc tcagcaaaga ccagtgcatt 1260
gtcatcagcg	gagagatgg	ctctgggaag	acagaaagcg cccacctgat tgttcacat 1320
ttgactttct	tgggaaaggc	caataatcag	accttgagag agaaaaattct acaagtcaac 1380
tccttggtgg	aagcctttgg	gaactcatgc	actgccatca atgacaactc gagccgtttt 1440
ggaaaatata	tggaaatgat	gtttacacca	actggagttg tgatgggggc aagaatctct 1500
gaatatctcc	tggaaaaatc	cagagttata	aaacaggcag cgagagagaa aaattttcat 1560
atattttact	atatttatgc	tggtcttcat	cacaaaaaga agctttctga tttcagactt 1620
cctgaggaaa	aacctcctag	gtacatagct	gatgaaactg gaagggtgat gcacgacata 1680
acttccaagg	agtcttacag	aagacaatto	gaagcaatto agcattgctt caggattata 1740
gggttcacgg	acaaagaggt	gcactcagtg	tacagaattt tggctgggat tttgaatatt 1800
gggaacattg	agttcgcagc	tatttcctct	caacatcaga ctgataaaa tgagggtccc 1860
aatgtgtaag	ctttgcaaaa	tgctgcctct	gttctgtgca ttagccctga agagctccag 1920
gaggecccca	cctcccactg	tggtgtcacc	cggggcgaga ccatcatccg tgccaacact 1980
gtagacaggg	ctgctggacg	tcgagacgcc	atgtccaaag ccctgtatgg gaggtcttcc 2040
agctggattg	tgaatcgcat	taatacactc	ctgcagccag acgaaaacat atgtagtgca 2100

-continued

```

ggagggtgaa tgaatgtgg gatcttgat atctttggat tcgagaattt tcagagaaat 2160
tcatttgagc agctctgcat aaacatcgcc aatgagcaaa tcagactata tttcaatcag 2220
catgtttttg ctcttgagca gatggaatat cagaatgaag gcattgatgc tatacccggtg 2280
gaatatgagg acaaccgccc gctcctggac atgttctctc agaaaccctt gggactgctt 2340
gcacttttgg atgaggaaaag tcgggtttccc caagcaactg accagaccct ggttgataaa 2400
tttgaagata atctacgatg caaatacttc tggaggccca aaggagtga actgtgcttt 2460
ggcattcagc attatgctgg aaaggattta tatgatgctt ctggggttct tgagaaaaat 2520
agagacactc tccctgccga tgtggttgtg gtcctgagaa cgtcagaaaa caagcttctt 2580
cagcagctct tctcaatccc tctgacaaa acaggtaatt tggccagac aagagctagg 2640
ataacagtgg cctcaagttc tttgctcca catttcagt ctgggaaagc caagggtggac 2700
actctggagg tgatacggca tccggaagaa accaccaaca tgaagaggca aactgtggct 2760
tcttacttcc ggtattctct gatggacctg ctctccaaaa tgggtgttgg acagccccac 2820
tttgtgcgct gcattaaacc caatgatgac cgagaggccc tgcagtcttc tcgagagagg 2880
gtgctggccc agctccgctc cacagggtt ctggagacag tcagcatccg ccgccagggc 2940
tattccacc gcacctttt tgaagaattt gtgaaaaggt attattactt ggcattcaca 3000
gcacatcaaa cacctcttgc tagcaaaag agctgtgtgg ctatcttga aaagtccaga 3060
ttagatcact ggggtgtggg aaaaacaaag gtttttctca aatattacca tgttgagcaa 3120
ytaaatttgc tgcttcgaga agtcataggc agagtgttg tgcgcaggc atataccaag 3180
gggtggcctg gagccaggag atacaaaag gtcagagaga agagagagaa gggagccatt 3240
gccatccagt cagcctggag aggatatgat gctcggagga aatttaagaa aataagcaac 3300
agaaggaatg agtctctgc tcataatcaa gcaggggcca cttcaacca aagcagtggg 3360
ccacattccc ccgtcgcagc aggtacgagg ggaagtgcg aggttcaaga ctgcagcgag 3420
cctggtgacc ataaagttct caggggtct gtacatcgta ggagccattc acaagcagaa 3480
tccaacaatg gccgtacaca gacttcaagc aactctctg ctgtcacaga gaaaatggg 3540
cattcacaag cccagagttc tccaaaagg tgcgatatct tcgcaggaca tgcaacaag 3600
gtagctggat atcttgatc caaagtaaat gtgtatcact ccttcagact catccaagtt 3660
cataggcatg aagctgtct cggctgcgt ggttgacca tccaaacttg a 3711

```

<210> SEQ ID NO 4

<211> LENGTH: 1236

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 4

```

Met Met Leu Gly Leu Glu Ser Leu Pro Asp Pro Thr Asp Thr Trp Glu
 1           5           10           15

```

```

Ile Ile Glu Thr Ile Gly Lys Gly Thr Tyr Gly Lys Val Tyr Lys Val
 20           25           30

```

```

Thr Asn Lys Arg Asp Gly Ser Leu Ala Ala Val Lys Ile Leu Asp Pro
 35           40           45

```

```

Val Ser Asp Met Asp Glu Glu Ile Glu Ala Glu Tyr Asn Ile Leu Gln
 50           55           60

```

```

Phe Leu Pro Asn His Pro Asn Val Val Lys Phe Tyr Gly Met Phe Tyr
 65           70           75           80

```

```

Lys Ala Asp His Cys Val Gly Gly Gln Leu Trp Leu Val Leu Glu Leu

```

-continued

85					90					95					
Cys	Asn	Gly	Gly	Ser	Val	Thr	Glu	Leu	Val	Lys	Gly	Leu	Leu	Arg	Cys
	100							105					110		
Gly	Gln	Arg	Leu	Asp	Glu	Ala	Met	Ile	Ser	Tyr	Ile	Leu	Tyr	Gly	Ala
	115					120						125			
Leu	Leu	Gly	Leu	Gln	His	Leu	His	Asn	Asn	Arg	Ile	Ile	His	Arg	Asp
	130					135					140				
Val	Lys	Gly	Asn	Asn	Ile	Leu	Leu	Thr	Thr	Glu	Gly	Gly	Val	Lys	Leu
	145					150					155				160
Val	Asp	Phe	Gly	Val	Ser	Ala	Gln	Leu	Thr	Ser	Thr	Arg	Leu	Arg	Arg
		165							170					175	
Asn	Thr	Ser	Val	Gly	Thr	Pro	Phe	Trp	Met	Ala	Pro	Glu	Val	Ile	Ala
			180					185					190		
Cys	Glu	Gln	Gln	Tyr	Asp	Ser	Ser	Tyr	Asp	Ala	Arg	Cys	Asp	Val	Trp
	195						200					205			
Ser	Leu	Gly	Ile	Thr	Ala	Ile	Glu	Leu	Gly	Asp	Gly	Asp	Pro	Pro	Leu
	210					215					220				
Phe	Asp	Met	His	Pro	Val	Lys	Thr	Leu	Phe	Lys	Ile	Pro	Arg	Asn	Pro
	225					230					235				240
Pro	Pro	Thr	Leu	Leu	His	Pro	Glu	Lys	Trp	Cys	Glu	Glu	Phe	Asn	His
			245						250					255	
Phe	Ile	Ser	Gln	Cys	Leu	Ile	Lys	Asp	Phe	Glu	Arg	Arg	Pro	Ser	Val
			260					265						270	
Thr	His	Leu	Leu	Asp	His	Pro	Phe	Ile	Lys	Gly	Val	His	Gly	Lys	Val
		275					280					285			
Leu	Phe	Leu	Gln	Lys	Gln	Leu	Ala	Lys	Val	Leu	Gln	Asp	Gln	Lys	His
	290					295					300				
Gln	Asn	Pro	Val	Ala	Lys	Thr	Arg	His	Glu	Arg	Met	His	Thr	Arg	Arg
	305					310					315				320
Pro	Tyr	His	Val	Glu	Asp	Ala	Glu	Lys	Tyr	Cys	Leu	Glu	Asp	Asp	Leu
			325						330					335	
Val	Asn	Leu	Glu	Val	Leu	Asp	Glu	Asp	Thr	Ile	Ile	His	Gln	Leu	Gln
		340						345					350		
Lys	Arg	Tyr	Ala	Asp	Leu	Leu	Ile	Tyr	Thr	Tyr	Val	Gly	Asp	Ile	Leu
		355					360					365			
Ile	Ala	Leu	Asn	Pro	Phe	Gln	Asn	Leu	Ser	Ile	Tyr	Ser	Pro	Gln	Phe
	370					375					380				
Ser	Arg	Leu	Tyr	His	Gly	Val	Lys	Arg	Ala	Ser	Asn	Pro	Pro	His	Ile
	385					390					395				400
Phe	Ala	Ser	Ala	Asp	Ala	Ala	Tyr	Gln	Cys	Met	Val	Thr	Leu	Ser	Lys
			405						410					415	
Asp	Gln	Cys	Ile	Val	Ile	Ser	Gly	Glu	Ser	Gly	Ser	Gly	Lys	Thr	Glu
			420					425					430		
Ser	Ala	His	Leu	Ile	Val	Gln	His	Leu	Thr	Phe	Leu	Gly	Lys	Ala	Asn
		435					440					445			
Asn	Gln	Thr	Leu	Arg	Glu	Lys	Ile	Leu	Gln	Val	Asn	Ser	Leu	Val	Glu
	450					455					460				
Ala	Phe	Gly	Asn	Ser	Cys	Thr	Ala	Ile	Asn	Asp	Asn	Ser	Ser	Arg	Phe
	465					470					475				480
Gly	Lys	Tyr	Leu	Glu	Met	Met	Phe	Thr	Pro	Thr	Gly	Val	Val	Met	Gly
			485						490					495	
Ala	Arg	Ile	Ser	Glu	Tyr	Leu	Leu	Glu	Lys	Ser	Arg	Val	Ile	Lys	Gln
			500					505					510		

-continued

Ala Ala Arg Glu Lys Asn Phe His Ile Phe Tyr Tyr Ile Tyr Ala Gly
 515 520 525
 Leu His His Gln Lys Lys Leu Ser Asp Phe Arg Leu Pro Glu Glu Lys
 530 535 540
 Pro Pro Arg Tyr Ile Ala Asp Glu Thr Gly Arg Val Met His Asp Ile
 545 550 555 560
 Thr Ser Lys Glu Ser Tyr Arg Arg Gln Phe Glu Ala Ile Gln His Cys
 565 570 575
 Phe Arg Ile Ile Gly Phe Thr Asp Lys Glu Val His Ser Val Tyr Arg
 580 585 590
 Ile Leu Ala Gly Ile Leu Asn Ile Gly Asn Ile Glu Phe Ala Ala Ile
 595 600 605
 Ser Ser Gln His Gln Thr Asp Lys Ser Glu Val Pro Asn Ala Glu Ala
 610 615 620
 Leu Gln Asn Ala Ala Ser Val Leu Cys Ile Ser Pro Glu Glu Leu Gln
 625 630 635 640
 Glu Ala Leu Thr Ser His Cys Val Val Thr Arg Gly Glu Thr Ile Ile
 645 650 655
 Arg Ala Asn Thr Val Asp Arg Ala Ala Asp Val Arg Asp Ala Met Ser
 660 665 670
 Lys Ala Leu Tyr Gly Arg Leu Phe Ser Trp Ile Val Asn Arg Ile Asn
 675 680 685
 Thr Leu Leu Gln Pro Asp Glu Asn Ile Cys Ser Ala Gly Gly Gly Met
 690 695 700
 Asn Val Gly Ile Leu Asp Ile Phe Gly Phe Glu Asn Phe Gln Arg Asn
 705 710 715 720
 Ser Phe Glu Gln Leu Cys Ile Asn Ile Ala Asn Glu Gln Ile Gln Tyr
 725 730 735
 Tyr Phe Asn Gln His Val Phe Ala Leu Glu Gln Met Glu Tyr Gln Asn
 740 745 750
 Glu Gly Ile Asp Ala Ile Pro Val Glu Tyr Glu Asp Asn Arg Pro Leu
 755 760 765
 Leu Asp Met Phe Leu Gln Lys Pro Leu Gly Leu Leu Ala Leu Leu Asp
 770 775 780
 Glu Glu Ser Arg Phe Pro Gln Ala Thr Asp Gln Thr Leu Val Asp Lys
 785 790 795 800
 Phe Glu Asp Asn Leu Arg Cys Lys Tyr Phe Trp Arg Pro Lys Gly Val
 805 810 815
 Glu Leu Cys Phe Gly Ile Gln His Tyr Ala Gly Lys Val Leu Tyr Asp
 820 825 830
 Ala Ser Gly Val Leu Glu Lys Asn Arg Asp Thr Leu Pro Ala Asp Val
 835 840 845
 Val Val Val Leu Arg Thr Ser Glu Asn Lys Leu Leu Gln Gln Leu Phe
 850 855 860
 Ser Ile Pro Leu Thr Lys Thr Gly Asn Leu Ala Gln Thr Arg Ala Arg
 865 870 875 880
 Ile Thr Val Ala Ser Ser Ser Leu Pro Pro His Phe Ser Ala Gly Lys
 885 890 895
 Ala Lys Val Asp Thr Leu Glu Val Ile Arg His Pro Glu Glu Thr Thr
 900 905 910
 Asn Met Lys Arg Gln Thr Val Ala Ser Tyr Phe Arg Tyr Ser Leu Met
 915 920 925

-continued

Asp Leu Leu Ser Lys Met Val Val Gly Gln Pro His Phe Val Arg Cys
 930 935 940
 Ile Lys Pro Asn Asp Asp Arg Glu Ala Leu Gln Phe Ser Arg Glu Arg
 945 950 955 960
 Val Leu Ala Gln Leu Arg Ser Thr Gly Ile Leu Glu Thr Val Ser Ile
 965 970 975
 Arg Arg Gln Gly Tyr Ser His Arg Ile Leu Phe Glu Glu Phe Val Lys
 980 985 990
 Arg Tyr Tyr Tyr Leu Ala Phe Thr Ala His Gln Thr Pro Leu Ala Ser
 995 1000 1005
 Lys Glu Ser Cys Val Ala Ile Leu Glu Lys Ser Arg Leu Asp His Trp
 1010 1015 1020
 Val Leu Gly Lys Thr Lys Val Phe Leu Lys Tyr Tyr His Val Glu Gln
 1025 1030 1035 1040
 Leu Asn Leu Leu Leu Arg Glu Val Ile Gly Arg Val Val Val Leu Gln
 1045 1050 1055
 Ala Tyr Thr Lys Gly Trp Leu Gly Ala Arg Arg Tyr Lys Lys Val Arg
 1060 1065 1070
 Glu Lys Arg Glu Lys Gly Ala Ile Ala Ile Gln Ser Ala Trp Arg Gly
 1075 1080 1085
 Tyr Asp Ala Arg Arg Lys Phe Lys Lys Ile Ser Asn Arg Arg Asn Glu
 1090 1095 1100
 Ser Ala Ala His Asn Gln Ala Gly Ala Thr Ser Asn Gln Ser Ser Gly
 1105 1110 1115 1120
 Pro His Ser Pro Val Ala Ala Gly Thr Arg Gly Ser Ala Glu Val Gln
 1125 1130 1135
 Asp Cys Ser Glu Pro Gly Asp His Lys Val Leu Arg Gly Ser Val His
 1140 1145 1150
 Arg Arg Ser His Ser Gln Ala Glu Ser Asn Asn Gly Arg Thr Gln Thr
 1155 1160 1165
 Ser Ser Asn Ser Pro Ala Val Thr Glu Lys Asn Gly His Ser Gln Ala
 1170 1175 1180
 Gln Ser Ser Pro Lys Gly Cys Asp Ile Phe Ala Gly His Ala Asn Lys
 1185 1190 1195 1200
 Val Ala Gly Tyr Leu Asp Ser Lys Val Asn Val Tyr His Ser Phe Arg
 1205 1210 1215
 Leu Ile Gln Val His Arg His Glu Ala Cys Leu Arg Leu Arg Gly Trp
 1220 1225 1230
 Thr Ile Gln Thr
 1235

<210> SEQ ID NO 5
 <211> LENGTH: 4034
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (1)...(4034)
 <223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 5

ttttcaacaa gatggagtct tgctctgttt cccagcctgt agtgcagtga cacagtcttg	60
gctcactgta acctctgcct cctgggttca agtgattctc ctgcctcagc etcctgagta	120
gctgggatta caggaaacat ctgtatggat tatttcacta taatcctatg atgcttggac	180
ttgaatcact tccagatccc acagacacct gggaaattat agagaccatt ggtaaaggca	240

-continued

cctatggcaa agtctacaag gtaactaaca agagagatgg gagcctggct gcagtgaana	300
ttctggatcc agtcagtgat atggatgaag aaattgaggc agaatacaac attttgagc	360
tccttcctaa tcatcccaat gttgtaaaagt tttatgggat gttttacaaa gcggatcact	420
gtgtaggggg acagctgtgg ctggctcctgg agctgtgtaa tgggggctca gtcacygagc	480
ttgtcaaaag tctactcaga tgtggccagc ggttgatga agcaatgatc tcatacatct	540
tgtacggggc cctcttgggc cttcagcatt tgcacaacaa ccgaatcatc caccgtgatg	600
tgaaggggaa taacattctt ctgacaacag aaggaggagt taagctcgtt gactttggtg	660
tttcagctca actcaccagt acacgtctgc ggagaaacac atctgttggc accccattct	720
ggatggcccc tgaggtcatt gcctgtgagc agcagtatga ctcttcctat gacgctcgt	780
gtgacgtctg gtccttgggg atcacagcta ttgaactggg ggatggagac cctccctct	840
ttgacatgca tcctgtgaaa acactcttta agattccaag aaatccctca cctactttac	900
ttcatccaga aaaatggtgt gaagaattca accactttat ttcacagtgt cttattaagg	960
atthtgaag gcgaccttc gtcacacatc tccttgacca cccatttatt aaaggagtac	1020
atggaaaagt tctgtttctg caaaaacagc tggccraggt tctccaagac cagaagcatc	1080
aaaatcctgt tgctaaaacc aggcagtga ggatgcatac cagaagacct tatcatgtgg	1140
aagatgtga aaaatactgc cttgaggatg atttgggtcaa cctagagggt ctggatgagg	1200
atacaattat ccatcagttg cagaaaagtt atgcagactt gctaatttac acatatgttg	1260
gagacatctt aattgcctta aacccttcc agaacttaag catatactct ccacagttt	1320
ccagacttta tcatggggtg aaacgcgcct ccaaycccc ccacatattt gcacagcag	1380
atgtgtctta ccagtgcag gttactctca gaaagacca gtgcattgtc atcagcggag	1440
agagtggctc tgggaagaca gaaagcgccc acctgattgt tcarcatthg actttcttgg	1500
gaaaggccaa taatcagacc ttgagagaga aaattctaca agtcaactcc ctggtggaag	1560
cctttgggaa ctcatgcact gccatcaatg acaactcgag ccgttttggg aaatatctgg	1620
aatgatgtt tacaccaact ggagtgtga tgggggcaag aatctctgaa tatctcctgg	1680
aaaaatccag agttataaaa caggcagcga gagagaaaaa ttttcatata ttttactata	1740
tttatgttg tcttcatcac caaaagaagc tttctgattt cagacttcct gaggaanaac	1800
ctcctaggtg catagctgat gaaactggaa ggtgatgca cgacataact tccaaggagt	1860
cttacagaag acaattcgaa gcaattcagc attgcttcag gattataggg ttcacggaca	1920
aagaggtgca ctcatgtac agaattttgg ctgggatttt gaatattggg aacattgagt	1980
tcgcagctat ttcctctcaa catcagactg ataaaagtga ggtgccaat gctgaagctt	2040
tgcaaaatgc tgccctctgt ctgtgcatta gccctgaaga gctccaggag gccctcacct	2100
cccactgtgt ggtcaccggg ggcgagacca tcatccgtgc caacactgta gacagggtg	2160
cggacgttgc agacgccatg tccaaagccc tgtatgggag gctcttcagc tggattgtga	2220
atcgcattaa tacactcctg cagccagacg aaacatatg tagtcagga ggtggaatga	2280
atgtggggat cttgatatac ttggatttcg agaattttca gaaaaattca tttgagcagc	2340
tctgcataaa catcgccaat gagcaaatcc agtactattt caatcagcat gttttgctc	2400
ttgagcagat ggaatatcag aatgaaggca ttgatgctat acccgtggaa tatgaggaca	2460
accgcccgtc cctggacatg ttcctccaga aaccctggg actgcttgca cttttggtg	2520
aggaaagctg gtttcccaa gcaactgacc agaccctggt tgataaattt gaagataatc	2580

-continued

tacgatgcaa atacttctgaggccaaaag gagggaact gtgctttggc attcagcatt	2640
atgctggaaa ggtattatat gatgcttctg gggttcttga gaaaaataga gacactctcc	2700
ctgccgatgt ggttggtgct ctgagaacgt cagaaaacaa gcttcttcag cagctcttct	2760
caatccctct gacccaaaaca ggtaatttgg ccagagacaag agctaggata acagtggcct	2820
caagttcttt gcctccacat ttcagtgtct ggaagccaa ggtggacact ctggaggtga	2880
tacggcatcc ggaagaaacc accaacatga agaggcaaac tgtggttct tacttcgggt	2940
attctctgat ggacctgtct tccaaaatgg tggttggaca gccccacttt gtgcgtgca	3000
ttaaacccaa tgatgaccga gaggccctgc agttctctcg agagagggtg ctggcccagc	3060
tccgctccac agggattctg gagacagtca gcaccgccc ccagggtat tcccaccgca	3120
tcctttttga agaatttgtg aaaaggtatt attacttggc attcacagca catcaaacac	3180
ctcttgctag caaagagagc tgtgtggcta tcttggaana gtccagatta gatcactggg	3240
tgctgggaaa aacaaagggt tttctcaaat attaccatgt tgagcaayta aatttgctgc	3300
ttcgagaagt cataggcaga gtggttgtgc tgcaggcata taccaagggg tggcttgag	3360
ccaggagata caaaaaggtc agagagaaga gagagaaggg agccattgcc atccagtcag	3420
cctggagagg atatgatgct cggaggaaat ttaagaaat aagcaacaga aggaatgagt	3480
ctgctgctca taatcaagca ggggccactt caaaccaag cagtgggcca cattcccccg	3540
tcgcagcagg tacgagggga agtgccagg ttcaagactg cagcgagcct ggtgaccata	3600
aagttctcag gggctctgta catcgtagga gccattcaca agcagaatcc aacaatggcc	3660
gtacacagac ttcaagcaac tctcctgctg tcacagagaa aaatgggcat tcacaagccc	3720
agagttctcc aaaagggtgc gatattctcg caggacatgc aaacaaggta gctggatctc	3780
ttgattccaa agtaaatgtg tatcactcct tcagactcat ccaagttcat aggcattgaag	3840
cttgctgctg gctgcgtggt tggaccatcc aaacttgaaa ctgttagtga tattttgaag	3900
tctttgagac aaaagcccag cttgctgaag aactttggtt cagtagagag acagggagggt	3960
acaggggaga gagaatcaaa agcctggaaa tttgctgctg agaataaatg ttagctgctc	4020
cctggngna aaaa	4034

<210> SEQ ID NO 6

<211> LENGTH: 2925

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 6

atgagaaggg cggggatcgg cgaggactcc aggctggggg tgcaggccca gccagggggc	60
gagccttctc cgggtcgggc ggggacagag cgctcccttg gaggcacca gggacctggc	120
cagccgtgca gctgccagg cgctatggcg agtgcggtca gggggtcgag gccgtggccc	180
cggctggggc tccagctcca gttcgcggcg ctgctgctcg ggacgtgag tccacaggtt	240
catactctca ggccagagaa cctcctgctg gtgtccacct tggatggaag tctccacgca	300
ctaagcaagc agacagggga cctgaagtgg actctgaggg atgatcccgat catcgaagga	360
ccaatgtacg tcacagaaat ggcctttctc tetgaccag cagatggcag cctgtacatc	420
ttggggaccc aaaaacaaca gggattaatg aaactgccat tcaccatccc tgagctgggt	480
catgcctctc cctgcgcgag ctctgatggg gtcttctaca caggccggaa gcaggatgcc	540
tggtttgtg tggacctga gtcaggggag acccagatga cactgaccac agaggggtccc	600
tccaccccc gcctctacat tggccgaaca cagtatacgg tcaccatgca tgaccaaga	660

-continued

gccccagccc tgcgctggaa caccacctac cgcgcctact cagcgccccc catggatggc	720
tcacctggga aatacatgag ccacctggcg tcctgcggga tgggcctgct gtcactgtg	780
gacccaggaa gcgggacggt gctgtggaca caggacctgg gcgtgcctgt gatgggcgtc	840
tacacctggc accaggacgg cctgcgccag ctgcccgcac tcacgctggc tcgagacact	900
ctgcatttcc tcgcccctcg ctggggccac atccgactgc ctgcctcagg cccccgggac	960
acagccaccc tcttctctac cttggacacc cagctgctaa tgacgctgta tgtggggaag	1020
gatgaaactg gcttctatgt ctytaagca ctggtccaca caggagtggc cctggtgcct	1080
cgtggactga ccttgccccc cgcagatggc cccaccacag atgaggtgac actccaagtc	1140
tcaggagagc gagagggtc acccagcact gctgttagat acccctcagg cagtgtggcc	1200
ctcccaagcc agtggctgct cattggacac cagcagctac cccagtcct gcacaccacc	1260
atgctgaggg tccatccac cctggggagt ggaactgcag agacaagacc tccagagaat	1320
acccaggccc cagccttctt cttggagcta ttgagcctga gccgagagaa actttgggac	1380
tccgagctgc atccagaaga aaaaactcca gactcttact tggggctggg accccaagac	1440
ctgctggcag ctagcctcac tctgtctctc ctgggagggg ggattctctt tgtgatgagg	1500
cagcaacagc cgcaggtggt ggagaagcag caggagaccc ccctggcacc tgcagacttt	1560
gctcacatct cccaggtatc ccagtcctc cactcggggg ccagccggag gagccagaag	1620
aggcttcaga gtccctcaaa gcaagcccag ccactcgacg accctgaagc tgagcaactc	1680
accgtagtgg ggaagatttc cttcaatccc aaggacgtgc tgggccgagg ggcaggcggg	1740
actttcgttt tccggggaca gtttgaggga cgggcagtggt ctgtcaagcg gctcctccgc	1800
gagtgccttg gcctggttcg cggggaagtt caactgctgc aggagtctga caggcaccac	1860
aacgtgctcc gctacttctg caccgagcgg ggaccccagt tccactacat tgccctggag	1920
ctctgcggcg cctccttgca ggagtacgta gaaaaccgg accctggatcg cgggggtctg	1980
gagcccaggg tcgtgtgca gcagctgatg tctggcctgg cccacctgca ctctttacac	2040
atagtgcacc gggacctgaa gccaggaaat attctcatca ccgggcctga cagccagggc	2100
ctgggcagag ttgtgtctct agacttcggc ctctgcaaga agctgcctgc tggccgctgt	2160
agcttcagcc tccactccgg catccccggc acggaaggct ggtggcgcc cagcttctg	2220
cagctcctgc caccagacag tccctaccagc gctgtggaca tcttctctgc aggtgcgtg	2280
ttctactacg tgctttctgg tggcagccac ccctttggag acagtcttta tcgccaggca	2340
aacatcctca caggggctcc ctgtctggct cactggagg aagaggtcca cgacaagggtg	2400
gttgcccggt accctggttg agccatgttg agccactgc cgcagccacg cccctctgcc	2460
ccccaggctc tggccacccc cttcttttgg agcagagcca agcaactcca gttcttccag	2520
gacgtcagtg actggttggg gaaggagtcc gagcaggagc ccctggtgag ggcactggag	2580
gcgggaggct gcgcagtggt ccgggacaac tggcacgagc acatctccat gccgctgcag	2640
acagatctga gaaagtccg gtccataag gggacatcag tgcgagacct gctccgtgct	2700
gtgaggaaca agaagcacca ctacaggag ctcccagttg aggtgcgaca ggcactcggc	2760
caagtcctgt atggtctcgt ccagtacttc acaaacgcgt tcccacggct gctcctccac	2820
acgcaccgag ccagtggag ctgcgcctct gagagcctct tctgcctta ctaccgcca	2880
gactcagagg ccaggaggcc atgccctggg gccacaggga ggtga	2925

-continued

<211> LENGTH: 974

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<220> FEATURE:

<221> NAME/KEY: VARIANT

<222> LOCATION: (1)...(974)

<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 7

```

Met Arg Arg Ala Gly Ile Gly Glu Asp Ser Arg Leu Gly Leu Gln Ala
 1             5             10            15
Gln Pro Gly Ala Glu Pro Ser Pro Gly Arg Ala Gly Thr Glu Arg Ser
      20            25            30
Leu Gly Gly Thr Gln Gly Pro Gly Gln Pro Cys Ser Cys Pro Gly Ala
      35            40            45
Met Ala Ser Ala Val Arg Gly Ser Arg Pro Trp Pro Arg Leu Gly Leu
      50            55            60
Gln Leu Gln Phe Ala Ala Leu Leu Leu Gly Thr Leu Ser Pro Gln Val
      65            70            75            80
His Thr Leu Arg Pro Glu Asn Leu Leu Leu Val Ser Thr Leu Asp Gly
      85            90            95
Ser Leu His Ala Leu Ser Lys Gln Thr Gly Asp Leu Lys Trp Thr Leu
      100           105           110
Arg Asp Asp Pro Val Ile Glu Gly Pro Met Tyr Val Thr Glu Met Ala
      115           120           125
Phe Leu Ser Asp Pro Ala Asp Gly Ser Leu Tyr Ile Leu Gly Thr Gln
      130           135           140
Lys Gln Gln Gly Leu Met Lys Leu Pro Phe Thr Ile Pro Glu Leu Val
      145           150           155           160
His Ala Ser Pro Cys Arg Ser Ser Asp Gly Val Phe Tyr Thr Gly Arg
      165           170           175
Lys Gln Asp Ala Trp Phe Val Val Asp Pro Glu Ser Gly Glu Thr Gln
      180           185           190
Met Thr Leu Thr Thr Glu Gly Pro Ser Thr Pro Arg Leu Tyr Ile Gly
      195           200           205
Arg Thr Gln Tyr Thr Val Thr Met His Asp Pro Arg Ala Pro Ala Leu
      210           215           220
Arg Trp Asn Thr Thr Tyr Arg Arg Tyr Ser Ala Pro Pro Met Asp Gly
      225           230           235           240
Ser Pro Gly Lys Tyr Met Ser His Leu Ala Ser Cys Gly Met Gly Leu
      245           250           255
Leu Leu Thr Val Asp Pro Gly Ser Gly Thr Val Leu Trp Thr Gln Asp
      260           265           270
Leu Gly Val Pro Val Met Gly Val Tyr Thr Trp His Gln Asp Gly Leu
      275           280           285
Arg Gln Leu Pro His Leu Thr Leu Ala Arg Asp Thr Leu His Phe Leu
      290           295           300
Ala Leu Arg Trp Gly His Ile Arg Leu Pro Ala Ser Gly Pro Arg Asp
      305           310           315           320
Thr Ala Thr Leu Phe Ser Thr Leu Asp Thr Gln Leu Leu Met Thr Leu
      325           330           335
Tyr Val Gly Lys Asp Glu Thr Gly Phe Tyr Val Xaa Lys Ala Leu Val
      340           345           350
His Thr Gly Val Ala Leu Val Pro Arg Gly Leu Thr Leu Ala Pro Ala
      355           360           365

```

-continued

Asp Gly Pro Thr Thr Asp Glu Val Thr Leu Gln Val Ser Gly Glu Arg
 370 375 380
 Glu Gly Ser Pro Ser Thr Ala Val Arg Tyr Pro Ser Gly Ser Val Ala
 385 390 395 400
 Leu Pro Ser Gln Trp Leu Leu Ile Gly His His Glu Leu Pro Pro Val
 405 410 415
 Leu His Thr Thr Met Leu Arg Val His Pro Thr Leu Gly Ser Gly Thr
 420 425 430
 Ala Glu Thr Arg Pro Pro Glu Asn Thr Gln Ala Pro Ala Phe Phe Leu
 435 440 445
 Glu Leu Leu Ser Leu Ser Arg Glu Lys Leu Trp Asp Ser Glu Leu His
 450 455 460
 Pro Glu Glu Lys Thr Pro Asp Ser Tyr Leu Gly Leu Gly Pro Gln Asp
 465 470 475 480
 Leu Leu Ala Ala Ser Leu Thr Ala Val Leu Leu Gly Gly Trp Ile Leu
 485 490 495
 Phe Val Met Arg Gln Gln Gln Pro Gln Val Val Glu Lys Gln Gln Glu
 500 505 510
 Thr Pro Leu Ala Pro Ala Asp Phe Ala His Ile Ser Gln Asp Ala Gln
 515 520 525
 Ser Leu His Ser Gly Ala Ser Arg Arg Ser Gln Lys Arg Leu Gln Ser
 530 535 540
 Pro Ser Lys Gln Ala Gln Pro Leu Asp Asp Pro Glu Ala Glu Gln Leu
 545 550 555 560
 Thr Val Val Gly Lys Ile Ser Phe Asn Pro Lys Asp Val Leu Gly Arg
 565 570 575
 Gly Ala Gly Gly Thr Phe Val Phe Arg Gly Gln Phe Glu Gly Arg Ala
 580 585 590
 Val Ala Val Lys Arg Leu Leu Arg Glu Cys Phe Gly Leu Val Arg Arg
 595 600 605
 Glu Val Gln Leu Leu Gln Glu Ser Asp Arg His Pro Asn Val Leu Arg
 610 615 620
 Tyr Phe Cys Thr Glu Arg Gly Pro Gln Phe His Tyr Ile Ala Leu Glu
 625 630 635 640
 Leu Cys Arg Ala Ser Leu Gln Glu Tyr Val Glu Asn Pro Asp Leu Asp
 645 650 655
 Arg Gly Gly Leu Glu Pro Glu Val Val Leu Gln Gln Leu Met Ser Gly
 660 665 670
 Leu Ala His Leu His Ser Leu His Ile Val His Arg Asp Leu Lys Pro
 675 680 685
 Gly Asn Ile Leu Ile Thr Gly Pro Asp Ser Gln Gly Leu Gly Arg Val
 690 695 700
 Val Leu Ser Asp Phe Gly Leu Cys Lys Lys Leu Pro Ala Gly Arg Cys
 705 710 715 720
 Ser Phe Ser Leu His Ser Gly Ile Pro Gly Thr Glu Gly Trp Met Ala
 725 730 735
 Pro Glu Leu Leu Gln Leu Leu Pro Pro Asp Ser Pro Thr Ser Ala Val
 740 745 750
 Asp Ile Phe Ser Ala Gly Cys Val Phe Tyr Tyr Val Leu Ser Gly Gly
 755 760 765
 Ser His Pro Phe Gly Asp Ser Leu Tyr Arg Gln Ala Asn Ile Leu Thr
 770 775 780
 Gly Ala Pro Cys Leu Ala His Leu Glu Glu Glu Val His Asp Lys Val

-continued

785	790	795	800
Val Ala Arg Asp Leu Val Gly Ala Met Leu Ser Pro Leu Pro Gln Pro	805	810	815
Arg Pro Ser Ala Pro Gln Val Leu Ala His Pro Phe Phe Trp Ser Arg	820	825	830
Ala Lys Gln Leu Gln Phe Phe Gln Asp Val Ser Asp Trp Leu Glu Lys	835	840	845
Glu Ser Glu Gln Glu Pro Leu Val Arg Ala Leu Glu Ala Gly Gly Cys	850	855	860
Ala Val Val Arg Asp Asn Trp His Glu His Ile Ser Met Pro Leu Gln	865	870	875
Thr Asp Leu Arg Lys Phe Arg Ser Tyr Lys Gly Thr Ser Val Arg Asp	885	890	895
Leu Leu Arg Ala Val Arg Asn Lys Lys His His Tyr Arg Glu Leu Pro	900	905	910
Val Glu Val Arg Gln Ala Leu Gly Gln Val Pro Asp Gly Phe Val Gln	915	920	925
Tyr Phe Thr Asn Arg Phe Pro Arg Leu Leu Leu His Thr His Arg Ala	930	935	940
Met Arg Ser Cys Ala Ser Glu Ser Leu Phe Leu Pro Tyr Tyr Pro Pro	945	950	955
Asp Ser Glu Ala Arg Arg Pro Cys Pro Gly Ala Thr Gly Arg	965	970	

<210> SEQ ID NO 8

<211> LENGTH: 2769

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 8

atgagaaggg cggggatcgg cgaggactcc aggctggggg tgcaggccca gccaggggcg	60
gagccttctc cgggtcgggc ggggacagag cgctcccttg gaggcacca gggacctggc	120
cagccgtgca gctgcccagg cgctatggcg agtgccgtca gggggtcgag gccgtggccc	180
cggctggggc tccagctcca gtctcgggcg ctgctgctcg ggacgctgag tccacaggtt	240
catactctca ggccagagaa cctcctgctg gtgtccacct tggatggaag tctccacgca	300
ctaagcaagc agacagggga cctgaagtgg actctgaggg atgatcccgat catcgaagga	360
ccaatgtacg tcacagaaat ggccttttct tctgaccag cagatggcag cctgtacatc	420
ttggggagcc aaaaacaaca gggattaatg aaactgccat tcaccatccc tgagctgggt	480
catgcctctc cctgccgag ctctgatggg gtcttttaca caggccggaa gcaggatgcc	540
tggtttgtgg tggacctga gtcaggggag acccagatga cactgaccac agaggggtccc	600
tccaccccc gcctctacat tggccgaaca cagtatacgg tcaccatgca tgaccaaga	660
gcccagccc tgcgtggaa caccacctac cgccgctact cagcgcccc catggatggc	720
tcacctggga aatacatgag ccacctggcg tcctcgggga tgggcctgct gctcactgtg	780
gaccagga gggggacgg gctgtggaca caggacctgg gcgtgcctgt gatgggcgtc	840
tacacctggc accaggacgg cctgcgccag ctgcccatc tcacgctggc tcgagacact	900
ctgcatttcc tcgcctccg ctggggccac atccgactgc ctgcctcagg cccccgggac	960
acagccacc tcttctctac ctggacacc cagctgctaa tgacgctgta tgtggggaag	1020
gatgaaactg gcttctatgt ctytaaagca ctggtccaca caggagtggc cctggtgcct	1080

-continued

```

cgtggactga cctggcccc cgcagatggc cccaccacag atgaggtgac actccaagtc 1140
tcaggagagc gagagggctc acccagcact gctgttagat acccctcagg cagtgtggcc 1200
ctcccaagcc agtgggtgct cattggacac cagcagctac cccagtcct gcacaccacc 1260
atgctgaggg tccatccccc cctggggagt ggaactgcag agacaagacc tccagagaat 1320
acccaggccc cagccttctt cttggagcaa cagccgcagg tggaggagaa gcagcaggag 1380
acccccctgg cactgcaga ctttgcac atctcccagg atgccagtc cctgcactcg 1440
ggggccagcc ggaggagcca gaagaggctt cagagtcctt caaagcaagc ccagccactc 1500
gacgacccctg aagctgagca actcaccgta gtggggaaga ttctcttcaa tcccaaggac 1560
gtgctgggccc gcggggcagg cgggactttc gttttccggg gacagtttga gggacgggca 1620
gtggctgtca agcggctcct ccgcgagtg cttggcctgg ttcggcggga agttcaactg 1680
ctgcaggagt ctgacaggca ccccaacgtg ctccgctact tctgcaccga gcgggggacc 1740
cagttccact acattgccct ggagctctgc cgggctcct tgcaggagta cgtagaaaac 1800
ccggacctgg atcgcggggg tctggagccc gaggtcgtgc tgcagcagct gatgtctggc 1860
ctggccccc tgcactctt acacatagtg caccgggacc tgaagccagg aaatattctc 1920
atcaccgggc ctgacagcca gggcctgggc agagtgtgc tctcagactt cggcctctgc 1980
aagaagctgc ctgtggcgg ctgtagcttc agcctccact ccggcatccc cggcacggaa 2040
ggctggatgg cggccgagct tctgcagctc ctgccaccag acagtcctac cagcgtgtg 2100
gacatcttct ctgcaggctg cgtgttctac tacgtgctt ctgggtggcag ccacccctt 2160
ggagacagtc ttatcgcca ggcaaacatc ctcacagggg ctccctgtct ggtcacctg 2220
gaggaagagg tccacgacaa ggtggttgcc cgggacctgg ttggagccat gttgagccca 2280
ctgccgcagc cagccccctc tgccccccag gtgctggccc accccttctt ttggagcaga 2340
gccaaagca tccagttctt ccaggacgtc agtgactggc tggagaagga gtccgagcag 2400
gagccccctg tgagggcact ggaggcggga ggctgcgcag tggctcggga caactggcac 2460
gagcacatct ccatgccgt gcagacagat ctgagaaagt tccggtccta taaggggaca 2520
tcagtgcgag acctgctccg tgctgtgagg aacaagaagc accactacag ggagctccca 2580
gttgagggtc gacaggcact cggccaagtc cctgatggct tcgtccagta cttcacaac 2640
cgcttcccc ggctgctcct ccacacgcac cgagccatga ggagctgcgc ctctgagagc 2700
ctcttctcgc cctactaccc gccagactca gaggccagga ggccatgcc tggggccaca 2760
gggaggtga 2769

```

```

<210> SEQ ID NO 9
<211> LENGTH: 922
<212> TYPE: PRT
<213> ORGANISM: homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(922)
<223> OTHER INFORMATION: Xaa = Any Amino Acid

```

```

<400> SEQUENCE: 9

```

```

Met Arg Arg Ala Gly Ile Gly Glu Asp Ser Arg Leu Gly Leu Gln Ala
 1             5             10             15

Gln Pro Gly Ala Glu Pro Ser Pro Gly Arg Ala Gly Thr Glu Arg Ser
                20             25             30

Leu Gly Gly Thr Gln Gly Pro Gln Gln Pro Cys Ser Cys Pro Gly Ala
 35             40             45

```

-continued

Met Ala Ser Ala Val Arg Gly Ser Arg Pro Trp Pro Arg Leu Gly Leu
 50 55 60

Gln Leu Gln Phe Ala Ala Leu Leu Leu Gly Thr Leu Ser Pro Gln Val
 65 70 75 80

His Thr Leu Arg Pro Glu Asn Leu Leu Leu Val Ser Thr Leu Asp Gly
 85 90 95

Ser Leu His Ala Leu Ser Lys Gln Thr Gly Asp Leu Lys Trp Thr Leu
 100 105 110

Arg Asp Asp Pro Val Ile Glu Gly Pro Met Tyr Val Thr Glu Met Ala
 115 120 125

Phe Leu Ser Asp Pro Ala Asp Gly Ser Leu Tyr Ile Leu Gly Thr Gln
 130 135 140

Lys Gln Gln Gly Leu Met Lys Leu Pro Phe Thr Ile Pro Glu Leu Val
 145 150 155 160

His Ala Ser Pro Cys Arg Ser Ser Asp Gly Val Phe Tyr Thr Gly Arg
 165 170 175

Lys Gln Asp Ala Trp Phe Val Val Asp Pro Glu Ser Gly Glu Thr Gln
 180 185 190

Met Thr Leu Thr Thr Glu Gly Pro Ser Thr Pro Arg Leu Tyr Ile Gly
 195 200 205

Arg Thr Gln Tyr Thr Val Thr Met His Asp Pro Arg Ala Pro Ala Leu
 210 215 220

Arg Trp Asn Thr Thr Tyr Arg Arg Tyr Ser Ala Pro Pro Met Asp Gly
 225 230 235 240

Ser Pro Gly Lys Tyr Met Ser His Leu Ala Ser Cys Gly Met Gly Leu
 245 250 255

Leu Leu Thr Val Asp Pro Gly Ser Gly Thr Val Leu Trp Thr Gln Asp
 260 265 270

Leu Gly Val Pro Val Met Gly Val Tyr Thr Trp His Gln Asp Gly Leu
 275 280 285

Arg Gln Leu Pro His Leu Thr Leu Ala Arg Asp Thr Leu His Phe Leu
 290 295 300

Ala Leu Arg Trp Gly His Ile Arg Leu Pro Ala Ser Gly Pro Arg Asp
 305 310 315 320

Thr Ala Thr Leu Phe Ser Thr Leu Asp Thr Gln Leu Leu Met Thr Leu
 325 330 335

Tyr Val Gly Lys Asp Glu Thr Gly Phe Tyr Val Xaa Lys Ala Leu Val
 340 345 350

His Thr Gly Val Ala Leu Val Pro Arg Gly Leu Thr Leu Ala Pro Ala
 355 360 365

Asp Gly Pro Thr Thr Asp Glu Val Thr Leu Gln Val Ser Gly Glu Arg
 370 375 380

Glu Gly Ser Pro Ser Thr Ala Val Arg Tyr Pro Ser Gly Ser Val Ala
 385 390 395 400

Leu Pro Ser Gln Trp Leu Leu Ile Gly His His Glu Leu Pro Pro Val
 405 410 415

Leu His Thr Thr Met Leu Arg Val His Pro Thr Leu Gly Ser Gly Thr
 420 425 430

Ala Glu Thr Arg Pro Pro Glu Asn Thr Gln Ala Pro Ala Phe Phe Leu
 435 440 445

Glu Gln Gln Pro Gln Val Val Glu Lys Gln Gln Glu Thr Pro Leu Ala
 450 455 460

Pro Ala Asp Phe Ala His Ile Ser Gln Asp Ala Gln Ser Leu His Ser

-continued

465	470	475	480
Gly Ala Ser Arg Arg Ser Gln Lys Arg Leu Gln Ser Pro Ser Lys Gln	485	490	495
Ala Gln Pro Leu Asp Asp Pro Glu Ala Glu Gln Leu Thr Val Val Gly	500	505	510
Lys Ile Ser Phe Asn Pro Lys Asp Val Leu Gly Arg Gly Ala Gly Gly	515	520	525
Thr Phe Val Phe Arg Gly Gln Phe Glu Gly Arg Ala Val Ala Val Lys	530	535	540
Arg Leu Leu Arg Glu Cys Phe Gly Leu Val Arg Arg Glu Val Gln Leu	545	550	555
Leu Gln Glu Ser Asp Arg His Pro Asn Val Leu Arg Tyr Phe Cys Thr	565	570	575
Glu Arg Gly Pro Gln Phe His Tyr Ile Ala Leu Glu Leu Cys Arg Ala	580	585	590
Ser Leu Gln Glu Tyr Val Glu Asn Pro Asp Leu Asp Arg Gly Gly Leu	595	600	605
Glu Pro Glu Val Val Leu Gln Gln Leu Met Ser Gly Leu Ala His Leu	610	615	620
His Ser Leu His Ile Val His Arg Asp Leu Lys Pro Gly Asn Ile Leu	625	630	635
Ile Thr Gly Pro Asp Ser Gln Gly Leu Gly Arg Val Val Leu Ser Asp	645	650	655
Phe Gly Leu Cys Lys Lys Leu Pro Ala Gly Arg Cys Ser Phe Ser Leu	660	665	670
His Ser Gly Ile Pro Gly Thr Glu Gly Trp Met Ala Pro Glu Leu Leu	675	680	685
Gln Leu Leu Pro Pro Asp Ser Pro Thr Ser Ala Val Asp Ile Phe Ser	690	695	700
Ala Gly Cys Val Phe Tyr Tyr Val Leu Ser Gly Gly Ser His Pro Phe	705	710	715
Gly Asp Ser Leu Tyr Arg Gln Ala Asn Ile Leu Thr Gly Ala Pro Cys	725	730	735
Leu Ala His Leu Glu Glu Glu Val His Asp Lys Val Val Ala Arg Asp	740	745	750
Leu Val Gly Ala Met Leu Ser Pro Leu Pro Gln Pro Arg Pro Ser Ala	755	760	765
Pro Gln Val Leu Ala His Pro Phe Phe Trp Ser Arg Ala Lys Gln Leu	770	775	780
Gln Phe Phe Gln Asp Val Ser Asp Trp Leu Glu Lys Glu Ser Glu Gln	785	790	795
Glu Pro Leu Val Arg Ala Leu Glu Ala Gly Gly Cys Ala Val Val Arg	805	810	815
Asp Asn Trp His Glu His Ile Ser Met Pro Leu Gln Thr Asp Leu Arg	820	825	830
Lys Phe Arg Ser Tyr Lys Gly Thr Ser Val Arg Asp Leu Leu Arg Ala	835	840	845
Val Arg Asn Lys Lys His His Tyr Arg Glu Leu Pro Val Glu Val Arg	850	855	860
Gln Ala Leu Gly Gln Val Pro Asp Gly Phe Val Gln Tyr Phe Thr Asn	865	870	875
Arg Phe Pro Arg Leu Leu Leu His Thr His Arg Ala Met Arg Ser Cys	885	890	895

-continued

Ala Ser Glu Ser Leu Phe Leu Pro Tyr Tyr Pro Pro Asp Ser Glu Ala
 900 905 910

Arg Arg Pro Cys Pro Gly Ala Thr Gly Arg
 915 920

<210> SEQ ID NO 10
 <211> LENGTH: 768
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 10

```

atgagaaggg cggggatcgg cgaggactcc aggctggggt tgcaggccca gccaggggcg    60
gagccttctc cgggtcgggc ggggacagag cgctcccttg gaggcaccca gggacctggc    120
cagccgtgca gctgcccagg cgctatggcg agtgcggtca gggggtcgag gccgtggccc    180
cggctggggc tccagctcca gtctcgggcg ctgctgctcg ggacgctgag tccacagggt    240
catactctca ggccagagaa cctcctgctg gtgtccacct tggatggaag tctccacgca    300
ctaagcaagc agacagggga cctgaagtgg actctgaggg atgatcccgat catcgaagga    360
ccaatgtacg tcacagaaat ggcctttctc tctgaccag cagatggcag cctgtacatc    420
ttggggaccc aaaaacaaca gggattaatg aaactgccat tcaccatccc tgagctggtt    480
catgcctctc cctgcgcgag ctctgatggg gtctcttaca caggccggaa gcaggatgcc    540
tggtttgtgg tggaccctga gtcaggggag acccagatga cactgaccac agagggtccc    600
tccaccccc gcctctacat tggccgaaca cagtatacgg tcaccatgca tgaccaaga    660
gccccagccc tgcgctggaa caccacctac cgccgctact cagcgcccc catggatggc    720
tcacctggga aatataaccc tccatgtgat ctccacacac cagactga    768

```

<210> SEQ ID NO 11
 <211> LENGTH: 255
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 11

```

Met Arg Arg Ala Gly Ile Gly Glu Asp Ser Arg Leu Gly Leu Gln Ala
  1           5           10           15

Gln Pro Gly Ala Glu Pro Ser Pro Gly Arg Ala Gly Thr Glu Arg Ser
  20           25           30

Leu Gly Gly Thr Gln Gly Pro Gly Gln Pro Cys Ser Cys Pro Gly Ala
  35           40           45

Met Ala Ser Ala Val Arg Gly Ser Arg Pro Trp Pro Arg Leu Gly Leu
  50           55           60

Gln Leu Gln Phe Ala Ala Leu Leu Leu Gly Thr Leu Ser Pro Gln Val
  65           70           75           80

His Thr Leu Arg Pro Glu Asn Leu Leu Leu Val Ser Thr Leu Asp Gly
  85           90           95

Ser Leu His Ala Leu Ser Lys Gln Thr Gly Asp Leu Lys Trp Thr Leu
  100          105          110

Arg Asp Asp Pro Val Ile Glu Gly Pro Met Tyr Val Thr Glu Met Ala
  115          120          125

Phe Leu Ser Asp Pro Ala Asp Gly Ser Leu Tyr Ile Leu Gly Thr Gln
  130          135          140

Lys Gln Gln Gly Leu Met Lys Leu Pro Phe Thr Ile Pro Glu Leu Val
  145          150          155          160

```

-continued

His	Ala	Ser	Pro	Cys	Arg	Ser	Ser	Asp	Gly	Val	Phe	Tyr	Thr	Gly	Arg
				165					170					175	
Lys	Gln	Asp	Ala	Trp	Phe	Val	Val	Asp	Pro	Glu	Ser	Gly	Glu	Thr	Gln
		180						185					190		
Met	Thr	Leu	Thr	Thr	Glu	Gly	Pro	Ser	Thr	Pro	Arg	Leu	Tyr	Ile	Gly
		195					200					205			
Arg	Thr	Gln	Tyr	Thr	Val	Thr	Met	His	Asp	Pro	Arg	Ala	Pro	Ala	Leu
		210				215				220					
Arg	Trp	Asn	Thr	Thr	Tyr	Arg	Arg	Tyr	Ser	Ala	Pro	Pro	Met	Asp	Gly
225					230					235				240	
Ser	Pro	Gly	Lys	Tyr	Asn	Pro	Pro	Cys	Asp	Leu	His	Thr	Pro	Asp	
			245						250					255	

What is claimed is:

1. An isolated nucleic acid molecule comprising the nucleotide sequence described in SEQ ID NO:6.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that:

(a) encodes the amino acid sequence shown in SEQ ID NO: 7; and

(b) hybridizes under stringent conditions with washing in 0.1xSSC/0.1% SDS at 68° C. to the nucleotide sequence of SEQ ID NO: 6 or the complement thereof.

3. An isolated nucleic acid molecule comprising the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 7.

4. A recombinant expression vector comprising the isolated nucleic acid molecule of claim 3.

5. A host cell comprising the recombinant expression vector of claim 4.

* * * * *

A handwritten capital letter 'R' in a cursive style, centered on the page.

This Page Blank (uspto)



US006476210B2

(12) **United States Patent**
Turner, Jr. et al.(10) **Patent No.: US 6,476,210 B2**
(45) **Date of Patent: Nov. 5, 2002**(54) **HUMAN KINASES AND
POLYNUCLEOTIDES ENCODING THE
SAME**(75) **Inventors:** C. Alexander Turner, Jr., The
Woodland, TX (US); Brian Mathur,
The Woodland, TX (US)(73) **Assignee:** Lexicon Genetics Incorporated, The
Woodlands, TX (US)(*) **Notice:** Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.(21) **Appl. No.:** 09/975,326(22) **Filed:** Oct. 11, 2001(65) **Prior Publication Data**

US 2002/0128458 A1 Sep. 12, 2002

Related U.S. Application Data(60) **Provisional application No.** 60/239,821, filed on Oct. 12,
2000.(51) **Int. Cl.⁷** C12N 15/54; C12N 9/12(52) **U.S. Cl.** 536/23.2; 435/194; 435/325;
435/252.3; 435/320.1(58) **Field of Search** 536/23.2; 435/194,
435/320.1, 252.3, 325(56) **References Cited****U.S. PATENT DOCUMENTS**

4,215,051	A	7/1980	Schroeder et al.
4,376,110	A	3/1983	David et al.
4,594,595	A	6/1986	Struckman
4,631,211	A	12/1986	Houghten
4,689,405	A	8/1987	Frank et al.
4,873,191	A	10/1989	Wagner et al.
4,946,778	A	8/1990	Ladner et al.
5,252,743	A	10/1993	Barrett et al.
5,272,057	A	12/1993	Smulson et al.
5,424,186	A	6/1995	Fodor et al.
5,445,934	A	8/1995	Fodor et al.
5,459,127	A	10/1995	Felgner et al.
5,556,752	A	9/1996	Lockhart
5,700,637	A	12/1997	Southern
5,723,326	A	3/1998	Kauffman et al.
5,744,305	A	4/1998	Fodor et al.
5,830,721	A	11/1998	Stemmer et al.
5,837,458	A	11/1998	Minshull et al.
5,869,336	A	2/1999	Meyer et al.
5,877,397	A	3/1999	Lonberg et al.
5,948,767	A	9/1999	Scheule et al.
6,075,181	A	6/2000	Kucherlapati et al.
6,110,490	A	8/2000	Thierry
6,117,679	A	9/2000	Stemmer
6,150,584	A	11/2000	Kucherlapati et al.

FOREIGN PATENT DOCUMENTS

WO WO00/73469 A2 * 12/2000

OTHER PUBLICATIONS

Alignment of AAB65619 with SEQ ID No: 2 and 4.*
Burgess HA, Martinez S, Reiner O. KIAA0369, doublecortin-like kinase, is expressed during brain development. J Neurosci Res. Nov. 15, 1999;58(4):567-75.*
Alignment of Q9JLM8 with SEQ ID No: 2 and 4.*
Prosrite analysis of SEQ ID No: 2 and 4.*
Bird et al, 1988, "Single-Chain Antigen-Binding Proteins", Science 242:423-426.
Bitter et al, 1987, "Expression and Secretion Vectors for Yeast" Methods in Enzymology 153:516-544.
Colbere-Garapin et al, 1981, "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol. 150:1-14.
Gautier et al, 1987 "α-DNA IV:α-anomeric and β-anomeric tetrathymidylates covalently linked to intercalating oxazolopyridocarbazole. Synthesis, physiochemical properties and poly (rA) binding", Nucleic Acids Research 15(16):6625-6641.
Gordon, 1989, "Transgenic Animals", International Review of Cytology, 115:117-229.
Greenspan et al, 1993, "Idiotypes: structure and immunogenicity", FASEB Journal 7:437-444.
Gu et al, 1994 "Deletion of a DNA Polymerase β Gene Segment in T Cells Using Cell Type-Specific Gene Targeting", Science 265:103-106.
Huse et al, 1989, "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", Science 246:1275-1281.
Huston et al, 1988, "Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*", Proc. Natl. Acad. Sci. USA 85:5879-5883.
Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and R Nase H", FEBS Letters 215(2):327-330.
Inoue et al, 1987, "Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides", Nucleic Acids Research 15(15):6131-6149.
Inouye & Inouye, 1985, "Up-promoter mutations in the Ipp gene of *Escherichia coli*", Nucleic Acids Research 13(9):3101-3110.
Janknecht et al, 1991, "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus", PNAS 88:8972-8976.
Kohler & Milstein, 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity", Nature 256:495-497.
Lakso et al, 1992, "Targeted oncogene activation by site-specific recombination in transgenic mice", Proc. Natl. Acad. Sci. USA 89:6232-6236.

(List continued on next page.)

Primary Examiner—Rebecca E. Prouty
Assistant Examiner—Sheridan Swope(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are disclosed that can be used in therapeutic, diagnostic, and pharmacogenomic applications.

4 Claims, No Drawings

OTHER PUBLICATIONS

- Lavitrano et al, 1989, "Sperm Cells and Vectors for Introducing Foreign DNA into Eggs: Genetic Transformation of Mice", *Cell* 57:717-723.
- Lo, 1983, "Transformation by Iontophoretic Microinjection of DNA: Multiple Integrations without Tandem Insertions", *Mol. & Cell. NBiology* 3(10):1803-1814.
- Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translations of mRNAs late after infection", *Proc. Natl. Acad. Sci. USA* 81:3655-3659.
- Lowy et al, 1980, "Isolation of Transforming DNA: Cloning the Hamster apt Gene", *Cell* 22:817-823.
- Morrison et al, 1984, "Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains", *Proc. Natl. Acad. Sci. USA* 81:6851-6855.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", *Proc. Natl. Acad. Sci. USA* 78(2):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies possessing novel effector functions" *Nature* 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", *J. of Immunology* 147:2429-2438.
- O'Hare et al, 1981, "Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase", *Proc. Natl. Acad. Sci. USA* 78(3): 1527-1531.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", *Gene* 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", *Proc. Natl. Acad. Sci. USA* 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", *J. Virol.* 46(2):584-593.
- Stein et al, 1988, "Physicochemical properties of phosphorothioate oligodeoxynucleotides", *Nucleic Acids Research* 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", *Proc. Natl. Acad. Sci. USA* 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", *Nature* 314:452-454.
- Thompson et al, 1989, "Germ Line Transmission and Expression of a Corrected HPRT Gene Produced by Gene Targeting in Embryonic Stem Cells", *Cell* 56:313-321.
- Van Der Putten et al, 1985, "Efficient insertion of genes into the mouse germ line via retroviral vectors", *Proc. Natl. Acad. Sci. USA* 82:6148-6152.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", *J. Biol. Chemistry* 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", *Nature* 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", *Cell* 11:223-232.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", *Proc. Natl. Acad. Sci. USA* 77(6):3567-3570.

* cited by examiner

1

HUMAN KINASES AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application No. 60/239,821, which was filed on Oct. 12, 2000, and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins sharing sequence similarity with animal kinases. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed genes, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed genes that can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of diseases and disorders, and cosmetic or nutraceutical applications.

2. BACKGROUND OF THE INVENTION

Kinases mediate the phosphorylation of a wide variety of proteins and compounds in the cell. Along with phosphatases, kinases are involved in a range of regulatory pathways. Given the physiological importance of kinases, they have been subject to intense scrutiny and are proven drug targets.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal kinases, including, but not limited to, serine-threonine kinases, calcium/calmodulin-dependent protein kinases, and mitogen activated kinases. Accordingly, the described NHPs encode novel kinases having homologues and orthologs across a range of phyla and species.

The novel human polynucleotides described herein, encode open reading frames (ORFs) encoding proteins of 766 and 765 amino acids in length (see respectively SEQ ID NOS: 2 and 4).

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof, that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and open reading frame or regulatory sequence replacement constructs) or to enhance the expression of the described NHPs (e.g., expression constructs that place the described polynucleotide under the control of a strong promoter system), and transgenic animals that express a NHP sequence, or "knock-outs" (which can be conditional) that do not express a functional NHP. Knock-out mice can be produced in several ways, one of which involves the use of mouse embryonic stem cells ("ES cells") lines that contain gene trap mutations in a murine homolog of at least one of the described NHPs. When the unique NHP

2

sequences described in SEQ ID NOS:1-4 are "knocked-out" they provide a method of identifying phenotypic expression of the particular gene as well as a method of assigning function to previously unknown genes. In addition, animals in which the unique NHP sequences described in SEQ ID NOS:1-4 are "knocked-out" provide a unique source in which to elicit antibodies to homologous and orthologous proteins that would have been previously viewed by the immune system as "self" and therefore would have failed to elicit significant antibody responses. To these ends, gene trapped knockout ES cells have been generated in murine homologs of the described NHPs.

Additionally, the unique NHP sequences described in SEQ ID NOS:1-4 are useful for the identification of protein coding sequence and mapping a unique gene to a particular chromosome. These sequences identify biologically verified exon splice junctions as opposed to splice junctions that may have been bioinformatically predicted from genomic sequence alone. The sequences of the present invention are also useful as additional DNA markers for restriction fragment length polymorphism (RFLP) analysis, and in forensic biology.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequence of the novel human ORFs encoding the described novel human kinase proteins.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPs described for the first time herein are novel proteins that are expressed in, inter alia, human cell lines and human fetal brain, brain, pituitary, spinal cord, testis, adipose, and esophagus cells. The described sequences were compiled from sequences available in GENBANK, and cDNAs generated from skeletal muscle, adipose, pituitary, cerebellum, and brain mRNA (Edge Biosystems, Gaithersburg, Md.).

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of an NHP that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of a NHP, or one of its domains (e.g., a receptor/ligand binding domain, accessory protein/

self-association domain, etc.) fused to another peptide or polypeptide; or (c) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing. As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel et al., eds., 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent expression product. Additionally, contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, *supra*), yet still encode a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. Nos. 5,837,458 or 5,723,323, both of which are herein incorporated by reference). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar to corresponding regions of SEQ ID NO:1 (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using default parameters).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP encoding polynucleotides. Such hybridization conditions can be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-4 can be used as a hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics, polymers, metal or metallized substrates, crystalline or polycrystalline substrates, etc.). Of particular note are spatially

addressable arrays (i.e., gene chips, microtiter plates, etc.) of oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-4, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, inter alia, U.S. Pat. Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-4 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-4.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-4 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

Probes consisting of sequences first disclosed in SEQ ID NOS:1-4 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

As an example of utility, the sequences first disclosed in SEQ ID NOS:1-4 can be utilized in microarrays or other assay formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences first disclosed in SEQ ID NOS:1-4 in silico and by comparing previously collected genetic databases and the disclosed sequences using computer software known to those in the art.

Thus the sequences first disclosed in SEQ ID NOS:1-4 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific oligonucleotide sequence(s) first disclosed in the SEQ ID NOS: 1-4. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (e.g., the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, Mich., etc.), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences can be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety that is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a

phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, supra.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

For example, the present sequences can be used in restriction fragment length polymorphism (RFLP) analysis to identify specific individuals. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification (as generally described in U.S. Pat. No. 5,272,057, incorporated herein by reference). In addition, the sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e., another DNA sequence that is unique to a particular individual). Actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing

PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene.

The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP sequence can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal sequence. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, immune disorders, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by

the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expression product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to NHP are likely to cross-react with a corresponding mutant NHP expression product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

An additional application of the described novel human polynucleotide sequences is their use in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Pat. Nos. 5,830,721, 5,837,458, 6,117,679, and 5,723,323, which are herein incorporated by reference in their entirety.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculovirus as described in U.S. Pat. No. 5,869,336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP sequence under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

Where, as in the present instance, some of the described NHP peptides or polypeptides are thought to be cytoplasmic or nuclear proteins (although processed forms or fragments can be secreted or membrane associated), expression systems can be engineered that produce soluble derivatives of a NHP (corresponding to a NHP extracellular and/or intracellular domains, or truncated polypeptides lacking one or more hydrophobic domains) and/or NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP domain to an IgFc), NHP antibodies, and anti-idiotypic antibodies (including Fab fragments) that can be used in therapeutic applications. Preferably, the above expression systems are engineered to allow the desired peptide or polypeptide to be recovered from the culture media.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP sequence (transcription factor inhibitors, antisense and ribozyme molecules, or open reading frame sequence or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of a NHP in the body. The use of engineered host cells and/or animals can offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor/ligand of a NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP or a protein interactive therewith. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products in vivo; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 THE NHP SEQUENCES

The cDNA sequences and corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing.

Expression analysis has provided evidence that the described NHPs can be expressed in a relatively narrow range of human tissues. In addition to serine-threonine kinases, the described NHPs also share significant similarity to a range of additional kinase families, including kinases associated with signal transduction, from a variety of phyla and species.

An additional application of the described novel human polynucleotide sequences is their use in the molecular

mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Pat. Nos. 5,830,721 and 5,837,458, which are herein incorporated by reference in their entirety.

NHP gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate NHP transgenic animals.

Any technique known in the art may be used to introduce a NHP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe and Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the NHP transgene in all their cells, as well as animals that carry the transgene in some, but not all their cells, i.e., mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al., 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that a NHP transgene be integrated into the chromosomal site of the endogenous NHP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NHP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NHP gene (i.e., "knockout" animals).

The transgene can also be selectively introduced into a particular cell type, thus inactivating the endogenous NHP gene in only that cell type, by following, for example, the teaching of Gu et al., 1994, Science, 265:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NHP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques that include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR. Samples of NHP gene-expressing tissue, may also be evaluated

immunocytochemically using antibodies specific for the NHP transgene product.

5.2 NHPS AND NHP POLYPEPTIDES

NHPS, NHP polypeptides, NHP peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include, but are not limited to, the generation of antibodies, as reagents in diagnostic assays, for the identification of other cellular gene products related to a NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease. Given the similarity information and expression data, the described NHPs can be targeted (by drugs, oligos, antibodies, etc.) in order to treat disease, or to therapeutically augment the efficacy of therapeutic agents.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP-encoding polynucleotides. The NHPs display initiator methionines that are present in DNA sequence contexts consistent with eucaryotic translation initiation sites. The NHPs do not display consensus signal sequences, which indicates that they may be cytoplasmic or possibly nuclear proteins, although they may also be secreted or membrane associated.

The NHP amino acid sequences of the invention include the amino acid sequences presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al., eds., Scientific American Books, New York, NY, herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and modify a NHP substrate, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but that result in a silent change, thus producing a functionally equivalent expression product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral

amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where the NHP peptide or polypeptide can exist, or has been engineered to exist, as a soluble or secreted molecule, the soluble NHP peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, in situ. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target expression product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. A NHP coding sequence can be

cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted sequence is expressed (e.g., see Smith et al., 1983, *J. Virol.* 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., see Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, *Methods in Enzymol.* 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the expression product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and expression products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the expression product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the NHP sequences described above can be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign

DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, *Cell* 22:817) genes, which can be employed in tk⁻, hgp^rt⁻ or ap^rt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, *Natl. Acad. Sci. USA* 77:3567; O'Hare, et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, *J. Mol. Biol.* 150:1); and hyg^r, which confers resistance to hygromycin (Santerre, et al., 1984, *Gene* 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al., allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-8976). In this system, the sequence of interest is subcloned into a vaccinia recombination plasmid such that the sequence's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Also encompassed by the present invention are fusion proteins that direct the NHP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHPs to antibody molecules or their Fab fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHP would also transport the NHP to the desired location within the cell. Alternatively targeting of NHP or its nucleic acid sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in "Liposomes: A Practical Approach", New, ed., Oxford University Press, New York and in U.S. Pat. Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective disclosures, which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHP to the target site or desired organ, where they cross the cell membrane and/or the nucleus where the NHP can exert its functional activity. This goal may be achieved by coupling of the NHP to a cytokine or other ligand that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. applications Ser. No. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes and can optionally be engineered to include nuclear localization.

5.3 ANTIBODIES TO NHP PRODUCTS

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention can be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP expression product. Additionally, such antibodies can be used in conjunction with gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with the NHP, a NHP peptide (e.g., one corresponding to a functional domain of a NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, chitosan, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and/or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl.*

Acad. Sci., 81:6851-6855; Neuberger et al., 1984, *Nature*, 312:604-608; Takeda et al., 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Pat. Nos. 6,075,181 and 5,877,397 and their respective disclosures, which are herein incorporated by reference in their entirety. Also encompassed by the present invention is the use of fully humanized monoclonal antibodies as described in U.S. Pat. No. 6,150,584 and respective disclosures, which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, *Science* 242:423-426; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 341:544-546) can be adapted to produce single chain antibodies against NHP expression products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments, which can be produced by pepsin digestion of the antibody molecule and the Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, *FASEB J* 7(5):437-444; and Nissinoff, 1991, *J. Immunol.* 147(8):2429-2438). For example antibodies that bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor/ligand can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind, activate, or neutralize a NHP, NHP receptor, or NHP ligand. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

Additionally given the high degree of relatedness of mammalian NHPs, the presently described knock-out mice (having never seen NHP, and thus never been tolerized to NHP) have a unique utility, as they can be advantageously applied to the generation of antibodies against the disclosed mammalian NHP (i.e., NHP will be immunogenic in NHP knock-out animals).

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1

<211> LENGTH: 2301

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atggccagca ccaggagtat cgagctggag cactttgagg aacgggacaa aaggccgagg 60
ccgggggtcgc ggagaggggc cccagctcc tccgggggca gcagcagctc gggccccaag 120
gggaacgggc tcatccccag tccggcgac agtgccact gcagcttcta ccgcacgcgg 180
accctgcagg ccctcagctc ggagaagaag gccagaagg cgcgcttcta ccggaacggg 240
gaccgctact tcaagggcct ggtgtttgcc atctccagcg accgcttcgg gtcyttcgat 300
gcgctcctca tagagctcac ccgctccctg tcggacaacg tgaacctgcc ccagggtgtc 360
cgcaactatct acaccatcga cggcagccgg aaggtcacca gcctggacga gctgctggaa 420
ggtgagagtt acgtgtgtgc atccaatgaa ccatttcgta aagtcgatta caccaaaaat 480
attaatccaa actggtctgt gaacatcaag ggtgggacat cccgagcgct ggtgtctgcc 540
tcctctgtga aaagtgaagt aaagaaagt aaagatttca tcaaacccaa gttagtgaat 600
gtgattcgaa gtggagtga gcttagaaaa gccgtgcgga tccttctgaa taaaagact 660
gtcatttctt ttgaacaagt cttacagat atcaccgaag ccattaaact agactcagga 720
gtcgtcaaga ggctctgcac cctggatgga aagcaggtta cttgtctgca agactttttt 780
ggtgatgacg atgtttttat tgcattgtga ccagaaaaat ttcgttatgc ccaagatgac 840
tttgtcctgg atcatagtga atgtcgtgtc ctgaagtcat cttattctcg atcctcagct 900
gttaagtatt ctggatccaa aagccctggg ccctctcgac gcagcaaatc accagcttca 960
gttaatggaa ctcccagcag ccaactttct actcctaaat ctacgaaatc ctccagttcc 1020
tctccaacta gtccaggaag ttccagagga ttaaagcaga tttctgctca tggcagatct 1080
tcttccaatg taaacggtgg acctgagctt gaccgttgca taagtctga aggtgtgaat 1140
ggaaacagat gctctgaatc atcaactctt cttgagaaat acaaaattgg aaaggtcatt 1200
ggtgatggca attttgcagt agtcaaagag tgtatagaca ggtccactgg aaaggagttt 1260
gccctaaaga ttatagacaa agccaaatgt tgtggaaagg aacacctgat tgagaatgaa 1320
gtgtcaatac tgcgccgagt gaaacatccc aatatcatta tgcgtgtcga ggagatggaa 1380
acagcaactg agctctttct ggtgatggaa ttggtcaaag gtggagatct ctttgatgca 1440
attacttcgt cgaccaagta cactgagaga gatggcagtg ccatggtgta caacttagcc 1500
aatgccctca ggtatctcca tggcctcagc atcgtgcaca gagacatcaa accagagaat 1560
ctcttggtgt gtgaatatcc tgatggaacc aagtctttga aactgggaga ctttgggctt 1620
gcgactgtgg tagaaggccc tttatacaca gtctgtggca ccccactta tgtggctcca 1680
gaaatcattg ctgaaactgg ctatggcctg aagggtgaca tttgggcagc tgggtgtgac 1740
acatacatat ttctctgtgg attcccacca ttccgaagtg agaacaatct ccaggagat 1800
ctcttcgacc agatcttggc tgggaagctg gagtttccgg cccctactg ggataacatc 1860
acggactctg ccaaggaatt aatcagtcga atgcttcagg taaatgttga agctcgggtg 1920
accgctgggc aaatcctgag tcacctctgg gtgtcagatg atgcctccca ggagaataac 1980
atgcaagctg aggtgacagg taaactaaaa cagcacttta ataatgcgct ccccaaacag 2040

```

-continued

```

aacagcacta ccaccggggt ctccgtcatc atgaacacgg ctctagataa ggaggggcag 2100
attttctgca gcaagcactg tcaagacagc ggcaggcctg ggatggagcc catctctcca 2160
gttcctccct cagtggagga gatccctgtg cctggggaag cagtcccggc cccaccct 2220
ccggaatctc ccacccccca ctgtcctccc gctgccccgg gtggtgagcg ggcaggaacc 2280
tggcgccgcc accgagactg a 2301

```

```

<210> SEQ ID NO 2
<211> LENGTH: 766
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 2

```

```

Met Ala Ser Thr Arg Ser Ile Glu Leu Glu His Phe Glu Glu Arg Asp
 1             5             10            15
Lys Arg Pro Arg Pro Gly Ser Arg Arg Gly Ala Pro Ser Ser Ser Gly
          20             25            30
Gly Ser Ser Ser Ser Gly Pro Lys Gly Asn Gly Leu Ile Pro Ser Pro
 35             40            45
Ala His Ser Ala His Cys Ser Phe Tyr Arg Thr Arg Thr Leu Gln Ala
 50             55            60
Leu Ser Ser Glu Lys Lys Ala Lys Lys Ala Arg Phe Tyr Arg Asn Gly
 65             70            75            80
Asp Arg Tyr Phe Lys Gly Leu Val Phe Ala Ile Ser Ser Asp Arg Phe
 85             90            95
Arg Ser Phe Asp Ala Leu Leu Ile Glu Leu Thr Arg Ser Leu Ser Asp
100            105           110
Asn Val Asn Leu Pro Gln Gly Val Arg Thr Ile Tyr Thr Ile Asp Gly
115            120           125
Ser Arg Lys Val Thr Ser Leu Asp Glu Leu Leu Glu Gly Glu Ser Tyr
130            135           140
Val Cys Ala Ser Asn Glu Pro Phe Arg Lys Val Asp Tyr Thr Lys Asn
145            150           155           160
Ile Asn Pro Asn Trp Ser Val Asn Ile Lys Gly Gly Thr Ser Arg Ala
165            170           175
Leu Ala Ala Ala Ser Ser Val Lys Ser Glu Val Lys Glu Ser Lys Asp
180            185           190
Phe Ile Lys Pro Lys Leu Val Thr Val Ile Arg Ser Gly Val Lys Pro
195            200           205
Arg Lys Ala Val Arg Ile Leu Leu Asn Lys Lys Thr Ala His Ser Phe
210            215           220
Glu Gln Val Leu Thr Asp Ile Thr Glu Ala Ile Lys Leu Asp Ser Gly
225            230           235           240
Val Val Lys Arg Leu Cys Thr Leu Asp Gly Lys Gln Val Thr Cys Leu
245            250           255
Gln Asp Phe Phe Gly Asp Asp Asp Val Phe Ile Ala Cys Gly Pro Glu
260            265           270
Lys Phe Arg Tyr Ala Gln Asp Asp Phe Val Leu Asp His Ser Glu Cys
275            280           285
Arg Val Leu Lys Ser Ser Tyr Ser Arg Ser Ser Ala Val Lys Tyr Ser
290            295           300
Gly Ser Lys Ser Pro Gly Pro Ser Arg Arg Ser Lys Ser Pro Ala Ser
305            310           315           320

```

-continued

Val	Asn	Gly	Thr	Pro	Ser	Ser	Gln	Leu	Ser	Thr	Pro	Lys	Ser	Thr	Lys	325	330	335	
Ser	Ser	Ser	Ser	Ser	Pro	Thr	Ser	Pro	Gly	Ser	Phe	Arg	Gly	Leu	Lys	340	345	350	
Gln	Ile	Ser	Ala	His	Gly	Arg	Ser	Ser	Ser	Asn	Val	Asn	Gly	Gly	Pro	355	360	365	
Glu	Leu	Asp	Arg	Cys	Ile	Ser	Pro	Glu	Gly	Val	Asn	Gly	Asn	Arg	Cys	370	375	380	
Ser	Glu	Ser	Ser	Thr	Leu	Leu	Glu	Lys	Tyr	Lys	Ile	Gly	Lys	Val	Ile	385	390	395	400
Gly	Asp	Gly	Asn	Phe	Ala	Val	Val	Lys	Glu	Cys	Ile	Asp	Arg	Ser	Thr	405	410	415	
Gly	Lys	Glu	Phe	Ala	Leu	Lys	Ile	Ile	Asp	Lys	Ala	Lys	Cys	Cys	Gly	420	425	430	
Lys	Glu	His	Leu	Ile	Glu	Asn	Glu	Val	Ser	Ile	Leu	Arg	Arg	Val	Lys	435	440	445	
His	Pro	Asn	Ile	Ile	Met	Leu	Val	Glu	Glu	Met	Glu	Thr	Ala	Thr	Glu	450	455	460	
Leu	Phe	Leu	Val	Met	Glu	Leu	Val	Lys	Gly	Gly	Asp	Leu	Phe	Asp	Ala	465	470	475	480
Ile	Thr	Ser	Ser	Thr	Lys	Tyr	Thr	Glu	Arg	Asp	Gly	Ser	Ala	Met	Val	485	490	495	
Tyr	Asn	Leu	Ala	Asn	Ala	Leu	Arg	Tyr	Leu	His	Gly	Leu	Ser	Ile	Val	500	505	510	
His	Arg	Asp	Ile	Lys	Pro	Glu	Asn	Leu	Leu	Val	Cys	Glu	Tyr	Pro	Asp	515	520	525	
Gly	Thr	Lys	Ser	Leu	Lys	Leu	Gly	Asp	Phe	Gly	Leu	Ala	Thr	Val	Val	530	535	540	
Glu	Gly	Pro	Leu	Tyr	Thr	Val	Cys	Gly	Thr	Pro	Thr	Tyr	Val	Ala	Pro	545	550	555	560
Glu	Ile	Ile	Ala	Glu	Thr	Gly	Tyr	Gly	Leu	Lys	Val	Asp	Ile	Trp	Ala	565	570	575	
Ala	Gly	Val	Ile	Thr	Tyr	Ile	Leu	Leu	Cys	Gly	Phe	Pro	Pro	Phe	Arg	580	585	590	
Ser	Glu	Asn	Asn	Leu	Gln	Glu	Asp	Leu	Phe	Asp	Gln	Ile	Leu	Ala	Gly	595	600	605	
Lys	Leu	Glu	Phe	Pro	Ala	Pro	Tyr	Trp	Asp	Asn	Ile	Thr	Asp	Ser	Ala	610	615	620	
Lys	Glu	Leu	Ile	Ser	Gln	Met	Leu	Gln	Val	Asn	Val	Glu	Ala	Arg	Cys	625	630	635	640
Thr	Ala	Gly	Gln	Ile	Leu	Ser	His	Pro	Trp	Val	Ser	Asp	Asp	Ala	Ser	645	650	655	
Gln	Glu	Asn	Asn	Met	Gln	Ala	Glu	Val	Thr	Gly	Lys	Leu	Lys	Gln	His	660	665	670	
Phe	Asn	Asn	Ala	Leu	Pro	Lys	Gln	Asn	Ser	Thr	Thr	Thr	Gly	Val	Ser	675	680	685	
Val	Ile	Met	Asn	Thr	Ala	Leu	Asp	Lys	Glu	Gly	Gln	Ile	Phe	Cys	Ser	690	695	700	
Lys	His	Cys	Gln	Asp	Ser	Gly	Arg	Pro	Gly	Met	Glu	Pro	Ile	Ser	Pro	705	710	715	720
Val	Pro	Pro	Ser	Val	Glu	Glu	Ile	Pro	Val	Pro	Gly	Glu	Ala	Val	Pro	725	730	735	
Ala	Pro	Thr	Pro	Pro	Glu	Ser	Pro	Thr	Pro	His	Cys	Pro	Pro	Ala	Ala				

-continued

740	745	750	
Pro Gly Gly Glu Arg Ala Gly Thr Trp Arg Arg His Arg Asp			
755	760	765	

<210> SEQ ID NO 3
 <211> LENGTH: 2298
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens
 <400> SEQUENCE: 3

```

atggccagca ccaggagtat cgagctggag cactttgagg aacgggacaa aagggcgcgg      60
ccgggggtcgc ggagaggggc ccccagctcc tccgggggca gcagcagctc gggccccaag      120
gggaacgggc tcatccccag tccggcgcac agtgcctact gcagcttcta ccgcacgcgg      180
accctgcagg ccctcagctc ggagaagaag gccagaagg cgcgcttcta ccggaacggg      240
gaccgtact tcaagggcct ggtgtttgcc atctccagcg accgcttcg gtcyttcgat      300
gcgctcctca tagagctcac ccgctccctg tcggacaacg tgaacctgcc ccaggggtgc      360
cgcaactatct acaccatcga cggcagccgg aaggtcacca gcctggacga gctgctggaa      420
ggtgagagtt acgtgtgtgc atccaatgaa ccatttcgta aagtcgatta caccaaaaat      480
attaatccaa actggtctgt gaacatcaag ggtgggacat cccgagcgct ggctgctgcc      540
tcctctgtga aaagtgaagt aaaagaaagt aaagatttca tcaaacccaa gttagtgaact      600
gtgattcgaa gtggagtga gcttagaaaa gccgtgcgga tccttctgaa taaaaagact      660
gctcattcct ttgaacaagt cttaacagat atcaccgaag ccattaaact agactcagga      720
gtcgtcaaga ggctctgcac cctggatgga aagcaggtta cttgtctgca agactttttt      780
ggtgatgacg atgtttttat tgcattgtga ccagaaaaat ttcgttatgc ccaagatgac      840
tttgtcctgg atcatagtga atgtcgtgtc ctgaagtcac cttattctcg atcctcagct      900
gttaagtatt ctggatccaa aagccctggg ccctctcgac gcagcaaatc accagcttca      960
gttaatggaa ctcccagcag ccaactttct actcctaaat ctacgaaatc ctccagttcc     1020
tctccaacta gtccaggaa tttcagagga ttaaagattt ctgctcatgg cagatcttct     1080
tccaatgtaa acggtggacc tgagcttgac cgttgcataa gtcctgaagg tgtgaatgga     1140
aacagatgct ctgaatcacc aactcttctt gagaaataca aaattggaaa ggtcattggg     1200
gatggcaatt ttgcagtagt caaagagtgt atagacaggt ccactggaaa ggagtttgcc     1260
ctaaagatta tagacaaagc caaatgttgt ggaagggaac acctgattga gaatgaagtg     1320
tcaatactgc gccgagtga acatcccaat atcattatgc tggtcgagga gatggaaaca     1380
gcaactgagc tctttctggt gatggaattg gtcaaagggt gagatctctt tgatgcaatt     1440
acttcgtcga ccaagtacac tgagagagat ggcagtgcca tgggtgtaca cttagccaat     1500
gcctcaggtt atctccatgg cctcagcacc gtgcacagag acatcaaacc agagaatctc     1560
ttgggtgtgt aatatcctga tggaaccaag tctttgaaac tgggagactt tgggcttgcg     1620
actgtggtag aaggcccttt atacacagtc tgtggcacac ccacttatgt ggctccagaa     1680
atcattgctg aaactggcta tggcctgaag gtggacattt gggcagctgg tgtgatcaca     1740
tacatacttc tctgtggatt cccaccattc cgaagtgaga acaatctcca ggaagatctc     1800
ttcgaccaga tcttggtggt gaagctggag tttccggccc cctactggga taacatcacg     1860
gactctgcca aggaattaat cagtcaaatg cttcaggtaa atgttgaagc tcggtgtacc     1920
gcggggacaaa tcctgagtca cccctgggtg tcagatgatg cctcccagga gaataacatg     1980
  
```

-continued

```

caagctgagg tgacaggtaa actaaaacag cactttaata atgcgctccc caaacagaac 2040
agcactacca ccgggggtctc cgtcatcatg aacacggctc tagataagga ggggcagatt 2100
ttctgcagca agcactgtca agacagcggc aggcctggga tggagcccat ctctccagtt 2160
cctccctcag tggaggagat ccctgtgcct ggggaagcag tcccggcccc caccctccg 2220
gaatctccca cccccactg tctctccgct gccccgggtg gtgagcgggc aggaacctgg 2280
cgccgccacc gagactga 2298

```

```

<210> SEQ ID NO 4
<211> LENGTH: 765
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 4

```

```

Met Ala Ser Thr Arg Ser Ile Glu Leu Glu His Phe Glu Glu Arg Asp
 1           5           10          15
Lys Arg Pro Arg Pro Gly Ser Arg Arg Gly Ala Pro Ser Ser Ser Gly
          20          25          30
Gly Ser Ser Ser Ser Gly Pro Lys Gly Asn Gly Leu Ile Pro Ser Pro
 35          40          45
Ala His Ser Ala His Cys Ser Phe Tyr Arg Thr Arg Thr Leu Gln Ala
 50          55          60
Leu Ser Ser Glu Lys Lys Ala Lys Lys Ala Arg Phe Tyr Arg Asn Gly
 65          70          75          80
Asp Arg Tyr Phe Lys Gly Leu Val Phe Ala Ile Ser Ser Asp Arg Phe
          85          90          95
Arg Ser Phe Asp Ala Leu Leu Ile Glu Leu Thr Arg Ser Leu Ser Asp
          100          105          110
Asn Val Asn Leu Pro Gln Gly Val Arg Thr Ile Tyr Thr Ile Asp Gly
          115          120          125
Ser Arg Lys Val Thr Ser Leu Asp Glu Leu Leu Glu Gly Glu Ser Tyr
          130          135          140
Val Cys Ala Ser Asn Glu Pro Phe Arg Lys Val Asp Tyr Thr Lys Asn
          145          150          155          160
Ile Asn Pro Asn Trp Ser Val Asn Ile Lys Gly Gly Thr Ser Arg Ala
          165          170          175
Leu Ala Ala Ala Ser Ser Val Lys Ser Glu Val Lys Glu Ser Lys Asp
          180          185          190
Phe Ile Lys Pro Lys Leu Val Thr Val Ile Arg Ser Gly Val Lys Pro
          195          200          205
Arg Lys Ala Val Arg Ile Leu Leu Asn Lys Lys Thr Ala His Ser Phe
          210          215          220
Glu Gln Val Leu Thr Asp Ile Thr Glu Ala Ile Lys Leu Asp Ser Gly
          225          230          235          240
Val Val Lys Arg Leu Cys Thr Leu Asp Gly Lys Gln Val Thr Cys Leu
          245          250          255
Gln Asp Phe Phe Gly Asp Asp Asp Val Phe Ile Ala Cys Gly Pro Glu
          260          265          270
Lys Phe Arg Tyr Ala Gln Asp Asp Phe Val Leu Asp His Ser Glu Cys
          275          280          285
Arg Val Leu Lys Ser Ser Tyr Ser Arg Ser Ser Ala Val Lys Tyr Ser
          290          295          300
Gly Ser Lys Ser Pro Gly Pro Ser Arg Arg Ser Lys Ser Pro Ala Ser
          305          310          315          320

```

-continued

Val Asn Gly Thr Pro Ser Ser Gln Leu Ser Thr Pro Lys Ser Thr Lys
 325 330 335
 Ser Ser Ser Ser Ser Pro Thr Ser Pro Gly Ser Phe Arg Gly Leu Lys
 340 345 350
 Ile Ser Ala His Gly Arg Ser Ser Ser Asn Val Asn Gly Gly Pro Glu
 355 360 365
 Leu Asp Arg Cys Ile Ser Pro Glu Gly Val Asn Gly Asn Arg Cys Ser
 370 375 380
 Glu Ser Ser Thr Leu Leu Glu Lys Tyr Lys Ile Gly Lys Val Ile Gly
 385 390 395 400
 Asp Gly Asn Phe Ala Val Val Lys Glu Cys Ile Asp Arg Ser Thr Gly
 405 410 415
 Lys Glu Phe Ala Leu Lys Ile Ile Asp Lys Ala Lys Cys Cys Gly Lys
 420 425 430
 Glu His Leu Ile Glu Asn Glu Val Ser Ile Leu Arg Arg Val Lys His
 435 440 445
 Pro Asn Ile Ile Met Leu Val Glu Glu Met Glu Thr Ala Thr Glu Leu
 450 455 460
 Phe Leu Val Met Glu Leu Val Lys Gly Gly Asp Leu Phe Asp Ala Ile
 465 470 475 480
 Thr Ser Ser Thr Lys Tyr Thr Glu Arg Asp Gly Ser Ala Met Val Tyr
 485 490 495
 Asn Leu Ala Asn Ala Leu Arg Tyr Leu His Gly Leu Ser Ile Val His
 500 505 510
 Arg Asp Ile Lys Pro Glu Asn Leu Leu Val Cys Glu Tyr Pro Asp Gly
 515 520 525
 Thr Lys Ser Leu Lys Leu Gly Asp Phe Gly Leu Ala Thr Val Val Glu
 530 535 540
 Gly Pro Leu Tyr Thr Val Cys Gly Thr Pro Thr Tyr Val Ala Pro Glu
 545 550 555 560
 Ile Ile Ala Glu Thr Gly Tyr Gly Leu Lys Val Asp Ile Trp Ala Ala
 565 570 575
 Gly Val Ile Thr Tyr Ile Leu Leu Cys Gly Phe Pro Pro Phe Arg Ser
 580 585 590
 Glu Asn Asn Leu Gln Glu Asp Leu Phe Asp Gln Ile Leu Ala Gly Lys
 595 600 605
 Leu Glu Phe Pro Ala Pro Tyr Trp Asp Asn Ile Thr Asp Ser Ala Lys
 610 615 620
 Glu Leu Ile Ser Gln Met Leu Gln Val Asn Val Glu Ala Arg Cys Thr
 625 630 635 640
 Ala Gly Gln Ile Leu Ser His Pro Trp Val Ser Asp Asp Ala Ser Gln
 645 650 655
 Glu Asn Asn Met Gln Ala Glu Val Thr Gly Lys Leu Lys Gln His Phe
 660 665 670
 Asn Asn Ala Leu Pro Lys Gln Asn Ser Thr Thr Thr Gly Val Ser Val
 675 680 685
 Ile Met Asn Thr Ala Leu Asp Lys Glu Gly Gln Ile Phe Cys Ser Lys
 690 695 700
 His Cys Gln Asp Ser Gly Arg Pro Gly Met Glu Pro Ile Ser Pro Val
 705 710 715 720
 Pro Pro Ser Val Glu Glu Ile Pro Val Pro Gly Glu Ala Val Pro Ala
 725 730 735

-continued

Pro	Thr	Pro	Pro	Glu	Ser	Pro	Thr	Pro	His	Cys	Pro	Pro	Ala	Ala	Pro
				740					745					750	

Gly	Gly	Glu	Arg	Ala	Gly	Thr	Trp	Arg	Arg	His	Arg	Asp
		755					760					765

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleotide sequence drawn from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that:

(a) encodes the amino acid sequence shown in SEQ ID NO:2; and

(b) hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1 or the complement thereof.

10 3. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO:2.

15 4. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO:4.

* * * * *

S

This Page Blank (uspto)



US006465632B1

(12) **United States Patent**
Walke et al.

(10) Patent No.: **US 6,465,632 B1**
(45) Date of Patent: **Oct. 15, 2002**

(54) **HUMAN PHOSPHATASES AND
POLYNUCLEOTIDES ENCODING THE
SAME**

(75) Inventors: **D. Wade Walke, Spring; John Scoville,
Houston; C. Alexander Turner, Jr.,
The Woodlands; Glenn Friedrich,
Houston; Alejandro Abuin, The
Woodlands; Brian Zambrowicz, The
Woodlands; Arthur T. Sands, The
Woodlands, all of TX (US)**

(73) Assignee: **Lexicon Genetics Incorporated, The
Woodlands, TX (US)**

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/877,730**

(22) Filed: **Jun. 8, 2001**

Related U.S. Application Data

(60) Provisional application No. 60/210,607, filed on Jun. 9,
2000.

(51) Int. Cl.⁷ **C07H 21/04; C12N 9/12**

(52) U.S. Cl. **536/23.2; 536/23.1; 536/23.5;
435/183; 435/194**

(58) Field of Search **435/183, 194;
536/23.1, 23.2; 530/350**

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,215,051 A	7/1980	Schroeder et al.	260/346.7
4,376,110 A	3/1983	David et al.	436/513
4,946,778 A	8/1990	Ladner et al.	435/69.6
5,723,323 A	3/1998	Kauffman et al.	435/172.3
5,830,721 A	11/1998	Stemmer et al.	435/172.1
5,837,458 A	11/1998	Minshull et al.	435/6
5,869,336 A	2/1999	Meyer et al.	435/348
5,939,271 A	8/1999	Tessier-Lavigne et al.	435/7.1
6,020,179 A	2/2000	Goli	435/196

OTHER PUBLICATIONS

Bird et al, 1988, "Single-Chain Antigen-Binding Proteins",
Science 242:423-426.

Bitter et al, 1987, "Expression and Secretion Vectors for
Yeast", Methods in Enzymology 153:516-544.

Colbere-Garapin et al, 1981, "A New Dominant Hybrid
Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol.
150:1-14.

Gautier et al, 1987, "α-DNA IV: α-anomeric and β-anomeric
tetrahydropyrimidylates covalently linked to intercalating
oxazopyridinecarbazole. Synthesis, physicochemical properties
and poly (rA) binding", Nucleic Acids Research
15(16):6625-6641.

Greenspan et al, 1993, "Idiotypes: structure and immuno-
genicity", FASEB Journal 7:437-444.

Huse et al, 1989, "Generation of a Large Combinatorial
Library of the Immunoglobulin Repertoire in Phage
Lambda", Science 246:1275-1281.

Huston et al, 1988, "Protein engineering of antibody bind-
ing sites: Recovery of specific activity in an anti-diogoxin
single-chain Fv analogue produced in *Escherichia coli*",
Proc. Natl. Acad. Sci. USA 85:5879-5883.

Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA
using modified oligonucleotide splints and R Nase H",
FEBS Letters 215(2):327-330.

Inoue et al, 1987, "Synthesis and hybridization studies on
two complementary nona(2'-O-methyl)ribonucleotides",
Nucleic Acids Research 15(15):6131-6149.

Inouye & Inouye, 1985, "Up-promoter mutations in the Ipp
gene of *Escherichia coli*", Nucleic Acids Research
13(9):3101-3110.

Janknecht et al, 1991, "Rapid and efficient purification of
native histidine-tagged protein expressed by recombinant
vaccinia virus", PNAS 88:8972-8976.

Kohler & Milstein, 1975, "Continuous cultures of fused
cells secreting antibody of predefined specificity", Nature
256:495-497.

Logan et al, 1984, "Adenovirus tripartite leader sequence
enhances translation of mRNAs late after infection", Proc.
Natl. Acad. Sci. USA 81:3655-3659.

Lowy et al, 1980, "Isolation of Transforming DNA: Cloning
the Hamster aprt Gene", Cell 22:817-823.

Morrison et al, 1984, "Chimeric human antibody molecules:
Mouse antigen-binding domains with human constant
region domains", Proc. Natl. Acad. Sci. USA 81:6851-6855.

Mulligan & Berg, 1981, "Selection for animal cells that
express the *Escherichia coli* gene coding for xanthine-gua-
nine phosphoribosyltransferase", Proc. Natl. Acad. Sci. USA
78(4):2072-2076.

Neuberger et al, 1984, "Recombinant antibodies possessing
novel effector functions", Nature 312:604-608.

Nisonoff, 1991, "Idiotypes: Concepts and Applications", J.
of Immunology 147:2429-2438.

O'Hare et al, 1981, "Transformation of mouse fibroblasts to
methotrexate resistance by a recombinant plasmid express-
ing a prokaryotic dihydrofolate reductase", Proc. Natl. Acad.
Sci. USA 78(3):1527-1531.

Ruther et al, 1983, "Easy identification of cDNA clones",
EMBO Journal 2(10): 1791-1794.

Santerre et al, 1984, "Expression of prokaryotic genes for
hygromycin B and G418 resistance as dominant-selection
markers in mouse L cells", Gene 30:147-156.

Sarin et al, 1988, "Inhibition of acquired immunodeficiency
syndrome virus by oligodeoxynucleoside methylphospho-
nates", Proc. Natl. Acad. Sci. USA 85:7448-7451.

(List continued on next page.)

Primary Examiner—Rebecca E. Prouty

(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are
disclosed that can be used in therapeutic, diagnostic, and
pharmacogenomic applications.

2 Claims, No Drawings

OTHER PUBLICATIONS

- Smith et al, 1983, "Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", J. Virol. 46(2):584-593.
- Stein et al, 1988, "Physiochemical properties of phosphorothioate oligodeoxynucleotides", Nucleic Acids Research 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", Proc. Natl. Acad. Sci. USA 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", Nature 314:452-454.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", J. Biol. Chemistry 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", Cell 11:223-232.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", Proc. Natl. Acad. Sci. USA 77(6):3567-3570.

1

HUMAN PHOSPHATASES AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application No. 60/210,607 which was filed on Jun. 9, 2000 and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins that share sequence similarity with animal phosphatases. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed genes, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed genes that can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of physiological disorders, or otherwise contributing to the quality of life.

2. BACKGROUND OF THE INVENTION

Membrane proteins can act as, inter alia, ligand receptors, signal transducers, neuronal guidance proteins, cell adhesion proteins, cell surface markers, and can also possess enzymatic functions such as the phosphorylation of substrates (i.e., kinase activity). Phosphatases mediate dephosphorylation of a wide variety of proteins and compounds in the cell. Often working in conjunction with kinases, phosphatases are involved in a regulating a wide range of biochemical and physiological pathways. Given the physiological importance of phosphatases, they have been subject to significant scrutiny and are good drug targets.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal immunoglobulin super family cell surface proteins, proteins that play a role in neuronal guidance (e.g., *nope*, *punc*, *unc*, and *neogenin*), phosphatases, netrin receptors, DCC (deleted in colon cancer) including, but not limited to tyrosine phosphatases, and cell adhesion molecules as homologues and orthologs across a range of phyla and species.

The novel human polynucleotides described herein, encode open reading frames (ORFs) encoding proteins of 1,069, 380, 904, 1150, 985, 991, 302, 826, 1072, 907, 712, 624, 547, 793, and 628 amino acids in length (see SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 respectively).

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof that compete with native NHPs, NHP peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described

2

NHP polynucleotides (e.g., expression constructs that place the described gene under the control of a strong promoter system). The present invention also includes both transgenic animals that express a NHP transgene, and NHP "knock-outs" (which can be conditional) that do not express a functional NHP. Knockout murine ES cells have been produced in a murine ortholog of the described NHPs.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP product activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequence of the novel human ORFs encoding the described novel human phosphatase proteins. SEQ ID NO:31 describes a NHP ORF and flanking sequences.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPs, described for the first time herein, are novel proteins that are expressed in, inter alia, human cell lines, and human brain, pituitary, kidney, testis, thyroid, adrenal gland, stomach, heart, uterus, placenta, mammary gland, adipose, esophagus, cervix, rectum, pericardium, ovary, fetal kidney and gene trapped human cells. The described sequences were compiled from gene trapped sequences in conjunction with sequences available in GENBANK, and cDNAs isolated from human testis and thyroid cDNA libraries (Edge Biosystems, Gaithersburg, Md.).

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of an NHP that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of a NHP, or one of its domains (e.g., a receptor/ligand binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing. As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g.,

hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, *supra*), yet still encode a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. Nos. 5,723,323 and 5,837,458 both of which are herein incorporated by reference). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar to corresponding regions of a sequence presented in the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using default parameters).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP encoding polynucleotides. Such hybridization conditions can be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length may partially overlap each other and/or the NHP sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described NHP polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 18, and preferably about 25, nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences may begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6×SSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as

NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences can be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, *Nucl. Acids Res.* 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, *FEBS Lett.* 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, *Nucl. Acids Res.* 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, *Molecular*

Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene.

The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, supra.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP

allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, immune disorders, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, AP-NHP or NHP-AP fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

An additional application of the described novel human polynucleotide sequences is their use in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Pat. Nos. 5,830,721 and 5,837,458 which are herein incorporated by reference in their entirety.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869,336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus hCMV immediate early gene, regulatable, viral (particularly retroviral LTR promoters) the early or late promoters of SV40 adenovirus, the lac system, the trp

system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

Where, as in the present instance, some of the described NHP peptides or polypeptides are thought to be cytoplasmic proteins, expression systems can be engineered that produce soluble derivatives of a NHP (corresponding to a NHP extracellular and/or intracellular domains, or truncated polypeptides lacking one or more hydrophobic domains) and/or NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP domain to an IgFc), NHP antibodies, and anti-idiotypic antibodies (including Fab fragments) that can be used in therapeutic applications. Preferably, the above expression systems are engineered to allow the desired peptide or polypeptide to be recovered from the culture media.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of a NHP in the body. The use of engineered host cells and/or animals can offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor/ligand of a NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP or a protein interactive therewith. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products in vivo; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also

encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 THE NHP SEQUENCES

The cDNA sequences and corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing. The NHP nucleotide sequences were obtained using the sequence information present in human gene trapped sequence tags and other cDNA sequences. Expression analysis has provided evidence that the described NHPs can be expressed in a wide variety of human tissues as well as gene trapped human cells. In addition to tyrosine phosphatases, the described NHPs also share significant similarity to a range of additional Ig super family proteins from a range of phyla and species. Given the physiological importance of protein phosphatases and other proteins that display structural relatedness to the described NHPs, such proteins have been subject to intense scrutiny as exemplified and discussed in U.S. Pat. Nos. 5,939,271 and 6,020,179 which describe a variety of uses and applications that can be applied to the described NHP sequences and which are herein incorporated by reference in their entirety.

Several polymorphisms were identified during sequencing such as an A-C transversion that can occur in the sequence region represented by, for example, nucleotide position 76 of SEQ ID NO:1 which can result in a L or M being present in the corresponding amino acid sequence at position, for example, 26 of SEQ ID NO:2, and an A-G transition that can occur in the sequence region represented by, for example, nucleotide position 706 of SEQ ID NO:1 which can result in a T or A being present in the corresponding amino acid sequence at, for example, position 236 of SEQ ID NO:2. The present invention contemplates sequences incorporating any of the above polymorphisms as well as all combinations and permutations thereof.

The gene encoding the described NHPs is apparently present on human chromosome 15 or human chromosome 3 (see GENBANK accession nos. AC012378 and AC012674). Accordingly, the described sequences are useful for identifying and mapping the coding regions of the human genome as well as identifying biologically validating functional exon splice junctions.

5.2 NHPs AND NHP POLYPEPTIDES

The described NHP products, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays (e.g., for cancer, neuronal abnormalities, Barbet-Biel Syndrome, etc.), the identification of other cellular gene products related to the NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease.

The Sequence Listing discloses the amino acid sequence encoded by the described NHP-encoding polynucleotides. The NHPs have initiator methionines in DNA sequence contexts consistent with eucaryotic translation initiation site, and display an apparent signal sequence near the N-terminus which indicates that the NHPs can be membrane associated, secreted, or cytoplasmic.

The NHP amino acid sequences of the invention include the amino acid sequences presented in the Sequence Listing

as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and modify a NHP substrate, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by a NHP nucleotide sequence described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where the NHP peptide or polypeptide can exist, or has been engineered to exist, as a soluble or secreted molecule, the soluble NHP peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, in situ. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with

recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP encoding polynucleotide sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the

appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, *Methods in Enzymol.* 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, *Cell* 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, *Natl. Acad. Sci. USA* 77:3567; O'Hare, et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, *J. Mol. Biol.* 150:1); and hyg^r, which confers resistance to hygromycin (Santerre, et al., 1984, *Gene* 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺ nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

5.3 Antibodies to NHP Products

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention can be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with the NHP, a NHP peptide (e.g., one corresponding to a functional domain of a NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983,

Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 341:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab)₂ frag-

ments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab)₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor/ligand can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind, activate, or neutralize a NHP, NHP receptor, or NHP ligand. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 31

<210> SEQ ID NO 1

<211> LENGTH: 3210

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atggcgccctc ctctcgacc cctcgcccg ctgcgaccgc cggggatgct gtccegcgc 60
ctctgtctcc tgctgmtgct cagtcctttg ccaggagtgt ggtgctttag cgaactgtct 120
tttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt ttagattgac 180
caggctcacg gagaagticc tattaaggtc acatgggtga aaaatggagc aaaaatgtct 240
gaaaataaac ggatcgaggt tctttctaac ggctctttat acatcagtga ggtggaaggc 300
aggcgaggag agcagtcoga tgaaggattt tatcagtgtc tggcaatgaa caaatatgga 360
gccattctta gtcaaaaagc tcattctgac ttatcaacta tttctgcatt tgaagtccag 420
ccaatttcca ctgaggtcca cgaagggtga gttgctgat ttgcattgca gatttcatcc 480
caccctcctg cagtcataac atgggagttc aatcggacaa ctctacctat gactatggac 540
aggataactg cctaccaaac aggagtattg cagatctatg atgtcagcca aagggtattct 600
ggaaattatc gttgtattgc tgccactgta gccaccgac gtaaaagtat ggaggcctcg 660
ctaactgtga ttccagctaa ggagtcaaaa tccttcacac caccarcaat tatagcaggt 720
ccacagaaca taacaacatc tcttcatcag actgtagttt tggaatgat ggccacagga 780

```

-continued

aatcccaaac caatcatttc ttggagccgc cttgatcaca aatccattga tgtctttaat	840
actcgggtac ttggaatgg taatctcatg atatctgatg tcaggctaca acatgctgga	900
gtatatgttt gtcggggccac taccctggc acacgcaact ttacagtgc tatggcaact	960
ttaactgtat tagctcctcc ttcatttggt gaatggccag aaagttaac aaggcctcga	1020
gctggcactg ctgatttgt gtgtcaggca gaaggaatcc cctctccaa gatgtcatgg	1080
ttgaaaaatg gaaggaagat acattcgaat ggtagaatta aaatgtacaa cagtaaatg	1140
gtaattaacc agattattcc tgaagatgat gctatttato agtgcattgg tgagaatagc	1200
caaggatcta ttttatctag agccagactg actgtagtga tgtcagaaga cagaccagat	1260
gctccctata atgtacatgc tgaaccatg tcaagctcag ccattctttt agcctgggag	1320
aggccacttt ataattcaga caaagtcatt gcctattctg tacactacat gaaagcagaa	1380
ggtttaata atgaagagta tcaagtagtc atcggaatg acacaactca ttatattatt	1440
gatgacttag agcctgccag caattatact ttctacattg tagcatatat gccaatggga	1500
gccagccaga tgtctgacca tgtgacacag aatactctag aggatgttcc cctgagacct	1560
cctgaaatta gtttgacaag tcgaagtccc actgatattc tcattctcctg gctgccaatc	1620
ccagccaaat atcggcgggg ccaagtggg ctgtatcgct tgtctttccg cctaagtact	1680
gagaattcaa tccaagtctt ggagctccc gggaccacgc atgagtacct ttggaaggc	1740
ctgaaacctg acagtgtcta cctggttcgg attactgctg ccaccagagt ggggctggga	1800
gagtcacag tatggacttc acataggacg cccaaagcta caagcgtgaa agcccttaag	1860
tctccagagt tgcatttgga gcctctgaac tgtaccacca tttctgtgag gtggcagcaa	1920
gatgtagagg acacagctgc tattcagggc tacaagctgt actacaagga agaaggcag	1980
caggagaatg ggccatttt cttggatacc aaggacctac tctatactct cagtggctta	2040
gaccccgaa gaaaatatca tgtgagactc ctggcttaca acaacataga cgatggctat	2100
caggcagatc agactgtcag cactccagga tgcgtgtctg ttcgtgatcg catggtccct	2160
cctccaccac caccaccaca tctctatgcy aaggctaaca cctcatcttc catcttctg	2220
cactggagga ggctgcatt caccgctgca caaatcatta actacacat ccgctgtaat	2280
cctgttgcc tgcagaatgc ttctttgggt ctgtaccttc aaacatcaga aactccatg	2340
ttggttcaag gtctagaacc aaacacaaa tacgaatttg ccgttcgatt acatgtggat	2400
cagctttcca gtccttgag ccctgtagtc taccattcta ctctccaga agcaccagca	2460
ggcccaccag ttggagtaaa agtgacatta atagaggatg acactgccct ggtttcttg	2520
aaacccctg atggcccaga aacagttgtg acccgctata ctattctata tgcatttagg	2580
aaggcctgga ttgcaggaga gtggcaggtc ttacaccgtg aaggggcaat aaccatggct	2640
ttgctagaaa acttggtagc aggaatgtg tacattgtca agatatctgc atccaatgag	2700
gtgggagaag gacccttttc aaattctgtg gagctggcag tacttccaaa ggaacctct	2760
gaatcaaatc agaggcccaa gcgttagat tctgctgatg ccaaagtta ttcaggatat	2820
taccatctg accaaaaatc aatgactggc attgctgtag gtgttgcat agccttgacc	2880
tgcattctca tctgtttct catcttgata taccgaagta aagccaggaa atcatctgct	2940
tccaagacgg cacagaatgg aactcaacag ttacctcgtc ccagtgcctc cttagctagt	3000
ggaaatgagg taggaaagaa cctggaagga gctgtaggaa atgaagaatc ttaaatgcca	3060
atgatcatgc caaacagctt cattgatgca aaggtactga gctgcgggat ttgctgcata	3120

-continued

agccgttctt ccattctccc tcctgtgtg tgtaaatgt acttcccca aaattgtatg 3180

ttgaatgtat tataccaata ctcttattaa 3210

<210> SEQ ID NO 2

<211> LENGTH: 1069

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

Met Ala Pro Pro Leu Arg Pro Leu Ala Arg Leu Arg Pro Pro Gly Met
1 5 10 15Leu Leu Arg Ala Leu Leu Leu Leu Leu Leu Ser Pro Leu Pro Gly
20 25 30Val Trp Cys Phe Ser Glu Leu Ser Phe Val Lys Glu Pro Gln Asp Val
35 40 45Thr Val Thr Arg Lys Asp Pro Val Val Leu Asp Cys Gln Ala His Gly
50 55 60Glu Val Pro Ile Lys Val Thr Trp Leu Lys Asn Gly Ala Lys Met Ser
65 70 75 80Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr Ile Ser
85 90 95Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe Tyr Gln
100 105 110Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys Ala His
115 120 125Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile Ser Thr
130 135 140Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile Ser Ser
145 150 155 160His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr Leu Pro
165 170 175Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu Gln Ile
180 185 190Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile Ala Ala
195 200 205Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr Val Ile
210 215 220Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile Ala Gly
225 230 235 240Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu Glu Cys
245 250 255Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg Leu Asp
260 265 270His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn Gly Asn
275 280 285Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr Val Cys
290 295 300Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met Ala Thr
305 310 315 320Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu Ser Leu
325 330 335Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala Glu Gly
340 345 350

Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His

-continued

355	360	365
Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln 370 375 380		
Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser 385 390 395 400		
Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu 405 410 415		
Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser 420 425 430		
Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys 435 440 445		
Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn 450 455 460		
Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile 465 470 475 480		
Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr 485 490 495		
Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln Asn Thr 500 505 510		
Leu Glu Asp Val Pro Leu Arg Pro Pro Glu Ile Ser Leu Thr Ser Arg 515 520 525		
Ser Pro Thr Asp Ile Leu Ile Ser Trp Leu Pro Ile Pro Ala Lys Tyr 530 535 540		
Arg Arg Gly Gln Val Val Leu Tyr Arg Leu Ser Phe Arg Leu Ser Thr 545 550 555 560		
Glu Asn Ser Ile Gln Val Leu Glu Leu Pro Gly Thr Thr His Glu Tyr 565 570 575		
Leu Leu Glu Gly Leu Lys Pro Asp Ser Val Tyr Leu Val Arg Ile Thr 580 585 590		
Ala Ala Thr Arg Val Gly Leu Gly Glu Ser Ser Val Trp Thr Ser His 595 600 605		
Arg Thr Pro Lys Ala Thr Ser Val Lys Ala Pro Lys Ser Pro Glu Leu 610 615 620		
His Leu Glu Pro Leu Asn Cys Thr Thr Ile Ser Val Arg Trp Gln Gln 625 630 635 640		
Asp Val Glu Asp Thr Ala Ala Ile Gln Gly Tyr Lys Leu Tyr Tyr Lys 645 650 655		
Glu Glu Gly Gln Gln Glu Asn Gly Pro Ile Phe Leu Asp Thr Lys Asp 660 665 670		
Leu Leu Tyr Thr Leu Ser Gly Leu Asp Pro Arg Arg Lys Tyr His Val 675 680 685		
Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln 690 695 700		
Thr Val Ser Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met Val Pro 705 710 715 720		
Pro Pro Pro Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser 725 730 735		
Ser Ile Phe Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile 740 745 750		
Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser 755 760 765		
Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val Gln Gly 770 775 780		

-continued

Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp
 785 790 795 800
 Gln Leu Ser Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro
 805 810 815
 Glu Ala Pro Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu
 820 825 830
 Asp Asp Thr Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr
 835 840 845
 Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile
 850 855 860
 Ala Gly Glu Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr Met Ala
 865 870 875 880
 Leu Leu Glu Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser
 885 890 895
 Ala Ser Asn Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu
 900 905 910
 Ala Val Leu Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg
 915 920 925
 Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp
 930 935 940
 Gln Lys Ser Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr
 945 950 955 960
 Cys Ile Leu Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg
 965 970 975
 Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro
 980 985 990
 Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu
 995 1000 1005
 Glu Gly Ala Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro
 1010 1015 1020
 Asn Ser Phe Ile Asp Ala Lys Val Leu Ser Cys Gly Ile Cys Cys Ile
 1025 1030 1035 1040
 Ser Arg Ser Ser Ile Pro Pro Pro Cys Val Cys Lys Met Tyr Phe Pro
 1045 1050 1055
 Gln Asn Cys Met Leu Asn Val Leu Tyr Gln Tyr Ser Tyr
 1060 1065

<210> SEQ ID NO 3
 <211> LENGTH: 1143
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 3

atggcgccctc ctctcgacc cctcgcccg ctgcgaccgc cggggatgct gctccgcgcg 60
 ctctctgctcc tgctgmtgct cagtcctttg ccaggagtgt ggtgctttag cgaactgtct 120
 tttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt tttagattgc 180
 caggctcagc gagaagttcc tattaagtc acatggttga aaatggagc aaaaatgtct 240
 gaaaataaac ggatcgaggt tctttctaac ggctctttat acatcagtga ggtggaaggc 300
 aggcgaggag agcagtcgga tgaaggattt tatcagtgtc tggcaatgaa caaatatgga 360
 gccattctta gtcaaaaagc tcatcttgcc ttatcaacta tttctgatt tgaagtccag 420
 ccaatttcca ctgaggtcca cgaaggtgga gttgctcgat ttgcatgcaa gatttcattc 480

-continued

```

caccctcctg cagtcataac atgggagttc aatcggacaa ctctacctat gactatggac 540
aggataactg cccatacaac aggagtattg cagatctatg atgtcagcca aagggtattct 600
ggaaattatc gttgtattgc tgccactgta gccacccgac gtaaaagtat ggaggcctcg 660
ctaactgtga ttccagctaa ggagtcaaaa tccttcacac caccarcaat tatagcaggt 720
ccacagaaca taacaacatc tcttcacag actgtagttt tggaatgcat gccacacagga 780
aatcccaaac caatcatttc ttggagccgc cttgatcaca aatccattga tgtctttaat 840
actcgggtac ttggaatgg taatctcatg atatctgatg tcaggctaca acatgctgga 900
gtatatgttt gtccggccac taccctggc acacgcaact ttacagttgc tatggcaact 960
ttaactgtat tagctcctcc ttcatttggt gaatggccag aaagttaac aaggcctcga 1020
gctggcactg ctgcatttgt gtgcaggca gaaggaatcc cctctcccaa gatgtcatgg 1080
ttgaaaaatg gaaggaagat acattcgaat ggtagaatta aaatgtacaa cagggttaaa 1140
taa 1143

```

<210> SEQ ID NO 4
 <211> LENGTH: 380
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 4

```

Met Ala Pro Pro Leu Arg Pro Leu Ala Arg Leu Arg Pro Pro Gly Met
 1          5          10          15
Leu Leu Arg Ala Leu Leu Leu Leu Leu Leu Ser Pro Leu Pro Gly
20          25          30
Val Trp Cys Phe Ser Glu Leu Ser Phe Val Lys Glu Pro Gln Asp Val
35          40          45
Thr Val Thr Arg Lys Asp Pro Val Val Leu Asp Cys Gln Ala His Gly
50          55          60
Glu Val Pro Ile Lys Val Thr Trp Leu Lys Asn Gly Ala Lys Met Ser
65          70          75          80
Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr Ile Ser
85          90          95
Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe Tyr Gln
100         105         110
Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys Ala His
115         120         125
Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile Ser Thr
130         135         140
Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile Ser Ser
145         150         155         160
His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr Leu Pro
165         170         175
Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu Gln Ile
180         185         190
Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile Ala Ala
195         200         205
Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr Val Ile
210         215         220
Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile Ala Gly
225         230         235         240
Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu Glu Cys
245         250         255

```

-continued

Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg Leu Asp
 260 265 270
 His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn Gly Asn
 275 280 285
 Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr Val Cys
 290 295 300
 Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met Ala Thr
 305 310 315 320
 Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu Ser Leu
 325 330 335
 Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala Glu Gly
 340 345 350
 Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His
 355 360 365
 Ser Asn Gly Arg Ile Lys Met Tyr Asn Arg Phe Lys
 370 375 380

<210> SEQ ID NO 5

<211> LENGTH: 2715

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 5

atggcgccctc ctctgcgacc cctcgcccggt ctgcgaccgc cggggatgct gctccgcgcg 60
 ctctgtctcc tgcgtggtgt cagtcctttg ccaggagtgt ggtgctttag cgaactgtct 120
 tttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt tttagattgc 180
 caggctcacg gagaagttcc tattaaggtc acatggttga aaaatggagc aaaaatgtct 240
 gaaaaataac ggatcgagggt tctttctaac ggctctttat acatcagtga ggtggaaggc 300
 aggcgaggag agcagtcgga tgaaggattt tatcagtgtc tggcaatgaa caaatatgga 360
 gccattctta gtcaaaaagc tcatcttgcc ttatcaacta tttctgcatt tgaagtccag 420
 ccaatttcca ctgagggtcca cgaagggtga gttgctcgat ttgcatgcaa gatttcattc 480
 caccctcctg cagtcataac atgggagttc aatcggacaa ctctacctat gactatggac 540
 aggataactg ccttaccaac aggagtattg cagatctatg atgtcagcca aagggtattc 600
 ggaaattatc gttgtattgc tgccactgta gccaccgcac gtaaaagtat ggaggcctcg 660
 ctaactgtga ttccagctaa ggagtcaaaa tccttcacaa caccarcaat tatagcaggt 720
 ccacagaaca taacaacatc tcttcacag actgtagttt tggaatgcat gccacagga 780
 aatcccaaac caatcatttc ttggagccgc cttgatcaca aatccattga tgcctttaat 840
 actcgggtac ttggaaatgg taatctcatg atatctgatg tcaggctaca acatgctgga 900
 gtatatgttt gtcggggccac tacccttggc acacgcaact ttacagttgc tatggcaact 960
 ttaactgtat tagctcctcc ttcatttgtt gaatggccag aaagtttaac aaggcctcga 1020
 gctggcactg ctcgatttgt gtgtcaggca gaaggaatcc cctctcccaa gatgtcatgg 1080
 ttgaaaaatg gaaggaagat acattcgaat ggtagaatta aaatgtacaa cagtaaattg 1140
 gtaattaacc agattattcc tgaagatgat gctatttata agtgcagggc tgagaatagc 1200
 caaggatcta ttttatctag agccagactg actgtagtga tgtcagaaga cagaccaggt 1260
 gctccctata atgtacatgc tgaaccatg tcaagctcag ccattctttt agcctgggag 1320
 aggccacttt ataattcaga caaagtcatt gcctattctg tacactacat gaaagcagaa 1380

-continued

ggtttaaata atgaagagta tcaagtagtc atcggaatg acacaactca ttatattatt 1440
 gatgacttag agcctgccag caattatact ttctacattg tagcatatat gccaatggga 1500
 gccagccaga tgtctgacca tgtgacacag aatactctag aggatgaccc cagaagaaaa 1560
 tatcatgtga gactcctggc ttacaacaac atagacgatg gctatcaggc agatcagact 1620
 gtcagcactc caggatgcgt gtctgttcgt gatcgcatgg tccctcctcc accaccaccc 1680
 caccatctct atgcgaaggc taacacctca tcttccatct tctgcatcg gaggaggcct 1740
 gcattcaccg ctgcacaaat cattaactac accatccgct gtaatcctgt tggcctgcag 1800
 aatgcttctt tggttctgta ccttcaaaca tcagaaactc acatgttggg tcaagggtta 1860
 gaaccaaaca ccaaatacga atttgccgtt cgattacatg tggatcagct ttccagtcct 1920
 tggagccctg tagtctacca ttctactctt ccagaagcac cagcaggccc accagttgga 1980
 gtaaaagtga cattaataga ggatgacact gccctgggtt cttggaaacc ccctgatggc 2040
 ccagaaacag ttgtgacccg ctatactatc ttatatgcat ctaggaaaggc ctggattgca 2100
 ggagagtggc aggtcttaca ccgtgaaggg gcaataacca tggctttgct agaaaacttg 2160
 gtagcaggaa atgtgtacat tgtcaagata tctgcatcca atgaggtggg agaaggaccc 2220
 ttttcaaatt ctgtggagct ggcagtactt ccaaaggaaa cctctgaatc aaatcagagg 2280
 cccaagcgtt tagattctgc tgatgccaaa gtttattcag gatattacca tctggaccaa 2340
 aaatcaatga ctggcattgc ttaggtgtt ggcatagcct tgacctgcat cctcatctgt 2400
 gttctcatct tgatataccg aagtaaagcc aggaatcat ctgcttccaa gacggcacag 2460
 aatggaactc aacagttacc tcgtaccagt gcctccttag ctagtggaaa tgaggtagga 2520
 aagaacctgg aaggagctgt aggaatgaa gaatctttaa tgccaatgat catgccaaac 2580
 agcttcattg atgcaaaggc actgagctgc gggatttgct gcataagccg ttcttcattt 2640
 cctctccct gtgtgtgtaa aatgtacttc ccccaaaatt gtatgttgaa tgtattatac 2700
 caatactctt attaa 2715

<210> SEQ ID NO 6

<211> LENGTH: 904

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 6

Met Ala Pro Pro Leu Arg Pro Leu Ala Arg Leu Arg Pro Pro Gly Met
 1 5 10 15

Leu Leu Arg Ala Leu Leu Leu Leu Leu Leu Ser Pro Leu Pro Gly
 20 25 30

Val Trp Cys Phe Ser Glu Leu Ser Phe Val Lys Glu Pro Gln Asp Val
 35 40 45

Thr Val Thr Arg Lys Asp Pro Val Val Leu Asp Cys Gln Ala His Gly
 50 55 60

Glu Val Pro Ile Lys Val Thr Trp Leu Lys Asn Gly Ala Lys Met Ser
 65 70 75 80

Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr Ile Ser
 85 90 95

Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe Tyr Gln
 100 105 110

Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys Ala His
 115 120 125

Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile Ser Thr

-continued

130	135	140
Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile Ser Ser		
145	150	155 160
His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr Leu Pro		
	165	170 175
Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu Gln Ile		
	180	185 190
Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile Ala Ala		
	195	200 205
Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr Val Ile		
	210	215 220
Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile Ala Gly		
	225	230 235 240
Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu Glu Cys		
	245	250 255
Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg Leu Asp		
	260	265 270
His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn Gly Asn		
	275	280 285
Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr Val Cys		
	290	295 300
Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met Ala Thr		
	305	310 315 320
Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu Ser Leu		
	325	330 335
Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala Glu Gly		
	340	345 350
Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His		
	355	360 365
Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln		
	370	375 380
Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser		
	385	390 395 400
Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu		
	405	410 415
Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser		
	420	425 430
Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys		
	435	440 445
Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn		
	450	455 460
Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile		
	465	470 475 480
Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr		
	485	490 495
Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln Asn Thr		
	500	505 510
Leu Glu Asp Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr		
	515	520 525
Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln Thr Val Ser Thr Pro		
	530	535 540
Gly Cys Val Ser Val Arg Asp Arg Met Val Pro Pro Pro Pro Pro		
	545	550 555 560

-continued

His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser Ser Ile Phe Leu His
 565 570 575
 Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile Ile Asn Tyr Thr Ile
 580 585 590
 Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser Leu Val Leu Tyr Leu
 595 600 605
 Gln Thr Ser Glu Thr His Met Leu Val Gln Gly Leu Glu Pro Asn Thr
 610 615 620
 Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp Gln Leu Ser Ser Pro
 625 630 635 640
 Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro Glu Ala Pro Ala Gly
 645 650 655
 Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu Asp Asp Thr Ala Leu
 660 665 670
 Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr Val Val Thr Arg Tyr
 675 680 685
 Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile Ala Gly Glu Trp Gln
 690 695 700
 Val Leu His Arg Glu Gly Ala Ile Thr Met Ala Leu Leu Glu Asn Leu
 705 710 715 720
 Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser Ala Ser Asn Glu Val
 725 730 735
 Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu Ala Val Leu Pro Lys
 740 745 750
 Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg Leu Asp Ser Ala Asp
 755 760 765
 Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp Gln Lys Ser Met Thr
 770 775 780
 Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr Cys Ile Leu Ile Cys
 785 790 795 800
 Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg Lys Ser Ser Ala Ser
 805 810 815
 Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro Arg Thr Ser Ala Ser
 820 825 830
 Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu Glu Gly Ala Val Gly
 835 840 845
 Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro Asn Ser Phe Ile Asp
 850 855 860
 Ala Lys Val Leu Ser Cys Gly Ile Cys Cys Ile Ser Arg Ser Ser Ile
 865 870 875 880
 Pro Pro Pro Cys Val Cys Lys Met Tyr Phe Pro Gln Asn Cys Met Leu
 885 890 895
 Asn Val Leu Tyr Gln Tyr Ser Tyr
 900

<210> SEQ ID NO 7
 <211> LENGTH: 3453
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 7

atggcgccctc ctctgcgacc cctcgcccggt ctgcgaccgc cggggatgct gctccgcgcg 60
 ctctgctcc tgctgctgct cagtcctttg ccaggagtgt ggtgctttag cgaactgtct 120

-continued

tttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt tttagattgc	180
caggctcacg gagaagttcc tattaaggtc acatggttga aaaatggagc aaaaatgtct	240
gaaaataaac ggatcgaggt tctttctaac ggctctttat acatcagtgga ggtggaaggc	300
agggcaggag agcagtcgga tgaaggattt tatcagtgct tggcaatgaa caaatatgga	360
gccattctta gtcaaaaagc tcatcttgcc ttatcaacta tttctgcatt tgaagtccag	420
ccaatttcca ctgagggtcca cgaagggtga gttgctcgat ttgcatgcaa gatttcattc	480
cacctctctg cagtcataac atgggagttc aatcggaaca ctctacctat gactatggac	540
aggataactg ccttaccac aggagttatt cagatctatg atgtcagcca aagggtattc	600
ggaaattatc gttgtattgc tggcactgta gccaccgac gtaaaagtat ggaggcctcg	660
ctaactgtga ttccagctaa ggagtcacaa tcctccaca caccaacaat tatagcaggt	720
ccacagaaca taacaacatc tcttcatcag actgtagttt tggaatgcat gccacagga	780
aatcccaaac caatcatttc ttggagccgc ctgtatcaca aatccattga tgtctttaat	840
actcgggtac ttggaaatgg taatctcatg atatctgatg tcaggctaca acatgctgga	900
gtatatgttt gtcgggcccac taccctggc acacgcaact ttacagttgc tatggcaact	960
ttaactgtat tagctctcc ttcatttgtt gaatggccag aaagttaac aaggcctcga	1020
gtgggactg ctgcatttgt gtgcaggca gaaggaaacc cctctcccaa gatgtcatgg	1080
ttgaaaatg gaaggagat acattcgaaat ggtagaatta aaatgtacaa cagtaaatg	1140
gtaattaacc agattattcc tgaagatgat gctatttacc agtgcattgc tgagaatagc	1200
caaggatcta ttttatctag agccagactg actgtagtga tgtcagaaga cagaccaggt	1260
gtccctata atgtacatgc tgaaccatg tcaagctcag ccattctttt agcctgggag	1320
aggccattt ataattcaga caaagtcatt gcctattctg tactactacat gaaagcagaa	1380
ggtttaata atgaagagta tcaagtagtc atcggaatg acacaactca ttatattatt	1440
gatgacttag agcctgccag caattatact ttctacattg tagcatatat gccaatggga	1500
gccagccaga tgtctgacca tgtgacacag aatactctag aggatgttcc cctgagacct	1560
cctgaaatta gtttgacaag tcgaagtccc actgatattc tcatctctg gctgccaatc	1620
ccagccaaat atcgcgggg ccaagtgtg ctgtatcgct tgtctttccg cctaagtact	1680
gagaattcaa tccaagttct ggagctccc gggaccacgc atgagtacct tttggaaggc	1740
ctgaaacctg acagtgtcta cctggttcg attactgctg ccaccagagt ggggtggga	1800
gagtcacag tatggacttc acataggag cccaaagcta caagcgtgaa agcccctaag	1860
tctccagagt tgcatttggg gcctctgaac tgtaccacca tttctgtgag gtggcagcaa	1920
gatgtagagg acacagctgc tattcagggc tacaagctgt actacaagga agaaggcgag	1980
caggagaatg ggccatttt cttggatacc aaggacctac tctatactct cagtggctta	2040
gaccccgaa gaaaatatca tgtgagactc ctggcttaca acaacataga cgtatgctat	2100
caggcagatc agactgtcag cactccagga tgcgtgtctg ttctgtatcg catggtccct	2160
cctccaccac caccocacca tctctatgcg aaggctaaca cctcatcttc catcttctg	2220
cactggagga ggcctgcatt caccgctgca caaatcatta actacacct cctgtgtaat	2280
cctgttggcc tgcagaatgc ttctttggtt ctgtaccttc aaacatcaga aactcacatg	2340
ttggttcaag gtctagaacc aaacacaaa tacgaatttg cgttcgatt acatgtggat	2400
cagctttcca gtccttgag cctgtatgc taccattcta ctctccaga agcaccagca	2460
ggccaccag ttggagtaaa agtgacatta atagaggatg acactgccct ggtttcttgg	2520

-continued

```

aaacccctg atggcccaga aacagttgtg acccgctata ctatcttata tgcctctagg 2580
aaggcctgga ttgcaggaga gtggcaggtc ttacaccgtg aaggggcaat aaccatggct 2640
ttgctagaaa acttggtagc aggaaatgtg tacattgtca agatatctgc atccaatgag 2700
gtgggagaag gaccttttcc aaattctgtg gagctggcag tacttccaaa ggaaacctct 2760
gaatcaaatc agaggcccaa gcgttttagat tctgctgatg ccaaagttta ttcaggatat 2820
taccatctgg accaaaaatc aatgactggc attgctgtag gtgttgccat agccttgacc 2880
tgcctcctca tctgtgttct catcttgata taccgaagta aagccaggaa atcatctgct 2940
tccaagacgg cagacaatgg aactcaacag ttacctcgta ccagtgcctc cttagctagt 3000
ggaaatgagg taggaaagaa cctggaagga gctgtaggaa atgaagaatc ttaaatgcca 3060
atgatcatgc caaacagctt cattgatgca aaggaggagaa ctgacctgat aattaatagc 3120
tatggctcta taattaaaaa caactctaag aaaaagtgtt ttttttcca agactcaaa 3180
aagatacaag ttgagcagcc tcaaagaaga tttactccag cggtctgctt ttaccagcca 3240
ggcaccactg tattaatcag tgatgaagac tcccctagct ccccgagtca gacaaccagc 3300
ttctcaagac cctttggtgt tgcagctgat acagaacatt cagcaaatag tgaaggcagc 3360
catgagactg gggattcttg gcggttttct catgagtcca acgatgagat acatctgtcc 3420
tcagttataa gtaccacacc ccccaacctc tga 3453

```

<210> SEQ ID NO 8

<211> LENGTH: 1150

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 8

```

Met Ala Pro Pro Leu Arg Pro Leu Ala Arg Leu Arg Pro Pro Gly Met
 1           5           10           15
Leu Leu Arg Ala Leu Leu Leu Leu Leu Leu Ser Pro Leu Pro Gly
 20          25          30
Val Trp Cys Phe Ser Glu Leu Ser Phe Val Lys Glu Pro Gln Asp Val
 35          40          45
Thr Val Thr Arg Lys Asp Pro Val Val Leu Asp Cys Gln Ala His Gly
 50          55          60
Glu Val Pro Ile Lys Val Thr Trp Leu Lys Asn Gly Ala Lys Met Ser
 65          70          75          80
Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr Ile Ser
 85          90          95
Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe Tyr Gln
100         105         110
Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys Ala His
115         120         125
Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile Ser Thr
130         135         140
Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile Ser Ser
145         150         155         160
His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr Leu Pro
165         170         175
Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu Gln Ile
180         185         190
Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile Ala Ala
195         200         205

```

-continued

Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr Val Ile
 210 215 220
 Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile Ala Gly
 225 230 235 240
 Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu Glu Cys
 245 250 255
 Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg Leu Asp
 260 265 270
 His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn Gly Asn
 275 280 285
 Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr Val Cys
 290 295 300
 Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met Ala Thr
 305 310 315 320
 Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu Ser Leu
 325 330 335
 Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala Glu Gly
 340 345 350
 Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His
 355 360 365
 Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln
 370 375 380
 Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser
 385 390 395 400
 Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu
 405 410 415
 Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser
 420 425 430
 Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys
 435 440 445
 Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn
 450 455 460
 Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile
 465 470 475 480
 Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr
 485 490 495
 Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln Asn Thr
 500 505 510
 Leu Glu Asp Val Pro Leu Arg Pro Pro Glu Ile Ser Leu Thr Ser Arg
 515 520 525
 Ser Pro Thr Asp Ile Leu Ile Ser Trp Leu Pro Ile Pro Ala Lys Tyr
 530 535 540
 Arg Arg Gly Gln Val Val Leu Tyr Arg Leu Ser Phe Arg Leu Ser Thr
 545 550 555 560
 Glu Asn Ser Ile Gln Val Leu Glu Leu Pro Gly Thr Thr His Glu Tyr
 565 570 575
 Leu Leu Glu Gly Leu Lys Pro Asp Ser Val Tyr Leu Val Arg Ile Thr
 580 585 590
 Ala Ala Thr Arg Val Gly Leu Gly Glu Ser Ser Val Trp Thr Ser His
 595 600 605
 Arg Thr Pro Lys Ala Thr Ser Val Lys Ala Pro Lys Ser Pro Glu Leu
 610 615 620

-continued

His Leu Glu Pro Leu Asn Cys Thr Thr Ile Ser Val Arg Trp Gln Gln
 625 630 635 640
 Asp Val Glu Asp Thr Ala Ala Ile Gln Gly Tyr Lys Leu Tyr Tyr Lys
 645 650 655
 Glu Glu Gly Gln Gln Glu Asn Gly Pro Ile Phe Leu Asp Thr Lys Asp
 660 665 670
 Leu Leu Tyr Thr Leu Ser Gly Leu Asp Pro Arg Arg Lys Tyr His Val
 675 680 685
 Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln
 690 695 700
 Thr Val Ser Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met Val Pro
 705 710 715 720
 Pro Pro Pro Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser
 725 730 735
 Ser Ile Phe Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile
 740 745 750
 Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser
 755 760 765
 Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val Gln Gly
 770 775 780
 Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp
 785 790 795 800
 Gln Leu Ser Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro
 805 810 815
 Glu Ala Pro Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu
 820 825 830
 Asp Asp Thr Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr
 835 840 845
 Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile
 850 855 860
 Ala Gly Glu Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr Met Ala
 865 870 875 880
 Leu Leu Glu Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser
 885 890 895
 Ala Ser Asn Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu
 900 905 910
 Ala Val Leu Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg
 915 920 925
 Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp
 930 935 940
 Gln Lys Ser Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr
 945 950 955 960
 Cys Ile Leu Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg
 965 970 975
 Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro
 980 985 990
 Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu
 995 1000 1005
 Glu Gly Ala Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro
 1010 1015 1020
 Asn Ser Phe Ile Asp Ala Lys Gly Gly Thr Asp Leu Ile Ile Asn Ser
 1025 1030 1035 1040
 Tyr Gly Pro Ile Ile Lys Asn Asn Ser Lys Lys Lys Trp Phe Phe Phe

-continued

1045	1050	1055
Gln Asp Ser Lys Lys Ile Gln Val Glu Gln Pro Gln Arg Arg Phe Thr 1060	1065	1070
Pro Ala Val Cys Phe Tyr Gln Pro Gly Thr Thr Val Leu Ile Ser Asp 1075	1080	1085
Glu Asp Ser Pro Ser Ser Pro Gly Gln Thr Thr Ser Phe Ser Arg Pro 1090	1095	1100
Phe Gly Val Ala Ala Asp Thr Glu His Ser Ala Asn Ser Glu Gly Ser 1105	1110	1115
His Glu Thr Gly Asp Ser Gly Arg Phe Ser His Glu Ser Asn Asp Glu 1125	1130	1135
Ile His Leu Ser Ser Val Ile Ser Thr Thr Pro Pro Asn Leu 1140	1145	1150

<210> SEQ ID NO 9
 <211> LENGTH: 2958
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 9

```

atggcgccctc ctctgcgacc cctcgcccgg ctgcgaccgc cggggatgct gctccgcgcg      60
ctcctgctcc tgctgmtgct cagtcctttg ccaggagtgt ggtgctttag cgaactgtct      120
tttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt tttagattgc      180
caggctcacg gagaagttcc tattaaggtc acatggttga aaaatggagc aaaaatgtct      240
gaaaataaac ggatcgaggt tctttctaac ggcctcttat acatcagtga ggtggaaggc      300
aggcgaggag agcagtcgga tgaaggattt tatcagtgct tggcaatgaa caaatatgga      360
gccattctta gtcaaaaagc tcattcttgc ttatcaacta tttctgcatt tgaagtcag      420
ccaatttcca ctgaggtcca cgaagggtga gttgctcgat ttgcatgcaa gatttcatcc      480
caccctcctg cagtcataac atgggagttc aatcggaaca ctctacctat gactatggac      540
aggataactg cctaccaaac aggagtattg cagatctatg atgtcagcca aagggattct      600
ggaaattatc gttgtattgc tgccactgta gccaccgac gtaaaagtat ggaggcctcg      660
ctaactgtga ttccagctaa ggagtcaaaa tccttcacca caccarcaat tatagcaggt      720
ccacagaaca taacaacatc tcttcacag actgtagttt tggaatgcat ggccacagga      780
aatcccaaac caatcatttc ttggagccgc cttgatcaca aatccattga tgtctttaat      840
actcgggtac ttggaatgg taatctcatg atatctgatg tcaggctaca acatgctgga      900
gtatatgttt gtcggggcac tacccctggc acacgcaact ttacagttgc tatggcaact      960
ttaactgtat tagctcctcc ttcatttgtt gaatggccag aaagttaac aaggcctcga      1020
gctggcactg ctcgatttgt gtgtcaggca gaaggaatcc cctctcccaa gatgtcatgg      1080
ttgaaaaatg gaaggaagat acattcgaat ggtagaatta aaatgtacaa cagtaaatg      1140
gtaattaacc agattattcc tgaagatgat gctatttata agtgcattgc tgagaatagc      1200
caaggatcta ttttatctag agccagactg actgtagtga tgtcagaaga cagaccagct      1260
gtccctata atgtacatgc tgaaccatg tcaagtcag ccattctttt agcctgggag      1320
aggccacttt ataattcaga caaagtcatt gcctattctg tacactacat gaaagcagaa      1380
ggtttaata atgaagagta tcaagtagtc atcggaatg acacaactca ttatattatt      1440
gatgacttag agcctgccag caattatact ttctacattg tagcatatat gccaatggga      1500
gccagccaga tgtctgacca tgtgacacag aatactctag aggatgaccc cagaagaaaa      1560

```

-continued

```

tatcatgtga gactcctggc ttacaacaac atagacgatg gctatcaggc agatcagact 1620
gtcagcactc caggatgcgt gtctgttcgt gatcgcatgg tccctcctcc accaccaccc 1680
caccatctct atgcgaaggc taacacctca tcttccatct tctgcaactg gaggaggcct 1740
gcattcaccg ctgcacaaat cattaactac accatccgct gtaatcctgt tggcctgcag 1800
aatgcttctt tggttctgta ccttcaaaca tcagaaactc acatgttggg tcaaggtcta 1860
gaaccaaaac ccaaatacga atttgccgtt cgattacatg tggatcagct ttccagtctt 1920
tggagccctg tagtctacca ttctactctt ccagaagcac cagcaggccc accagttgga 1980
gtaaaagtga cattaataga ggatgacact gccctggttt cttggaaacc cctgatggc 2040
ccagaacacg ttgtgacctg ctatactatc ttatatgcat ctaggaaaggc ctggattgca 2100
ggagagtggc aggtcttaca ccgtgaaggg gcaataacca tggctttgct agaaaacttg 2160
gtagcaggaa atgtgtacat tgtcaagata tctgcatcca atgaggtggg agaaggaccc 2220
ttttcaaatt ctgtggagct ggcagtactt ccaaaggaaa cctctgaatc aaatcagagg 2280
cccaagcgtt tagattctgc tgatgccaaa gtttattcag gatattacca tctggaccaa 2340
aaatcaatga ctggcattgc tgtaggtgtt ggcatagcct tgacctgcat cctcatctgt 2400
gttctcatct tcatataccg aagtaaagcc aggaaatcat ctgcttccaa gacggcacag 2460
aatggaactc aacagttacc tcgtaccagt gccctccttag ctagtggaaa tgaggtagga 2520
aagaacctgg aaggagctgt aggaatgaa gaatctttaa tgccaatgat catgccaaac 2580
agcttcattg atgcaaaggg aggaactgac ctgataatta atagctatgg tcctataatt 2640
aaaaacaact ctaagaaaaa gtggtttttt ttccaagact caaagaagat acaagttgag 2700
cagcctcaaa gaagatttac tccagcggtc tgcttttacc agccaggcac cactgtatta 2760
atcagtgatg aagactcccc tagtcccca ggtcagacaa ccagcttctc aagacccttt 2820
gggtgtgcag ctgatacaga acattcagca aatagtgaag gcagccatga gactggggat 2880
tctggcggtt tttctcatga gtccaacgat gagatacatc tgcctcagt tataagtacc 2940
acacccccca acctctga 2958

```

<210> SEQ ID NO 10

<211> LENGTH: 985

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 10

```

Met Ala Pro Pro Leu Arg Pro Leu Ala Arg Leu Arg Pro Pro Gly Met
 1           5           10          15
Leu Leu Arg Ala Leu Leu Leu Leu Leu Leu Ser Pro Leu Pro Gly
 20          25          30
Val Trp Cys Phe Ser Glu Leu Ser Phe Val Lys Glu Pro Gln Asp Val
 35          40          45
Thr Val Thr Arg Lys Asp Pro Val Val Leu Asp Cys Gln Ala His Gly
 50          55          60
Glu Val Pro Ile Lys Val Thr Trp Leu Lys Asn Gly Ala Lys Met Ser
 65          70          75          80
Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr Ile Ser
 85          90          95
Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe Tyr Gln
100         105         110
Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys Ala His

```


-continued

115	120	125
Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile Ser Thr 130	135	140
Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile Ser Ser 145	150	155 160
His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr Leu Pro 165	170	175
Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu Gln Ile 180	185	190
Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile Ala Ala 195	200	205
Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr Val Ile 210	215	220
Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile Ala Gly 225	230	235 240
Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu Glu Cys 245	250	255
Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg Leu Asp 260	265	270
His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn Gly Asn 275	280	285
Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr Val Cys 290	295	300
Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met Ala Thr 305	310	315 320
Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu Ser Leu 325	330	335
Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala Glu Gly 340	345	350
Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His 355	360	365
Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln 370	375	380
Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser 385	390	395 400
Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu 405	410	415
Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser 420	425	430
Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys 435	440	445
Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn 450	455	460
Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile 465	470	475 480
Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr 485	490	495
Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln Asn Thr 500	505	510
Leu Glu Asp Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr 515	520	525
Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln Thr Val Ser Thr Pro 530	535	540

-continued

Gly Cys Val Ser Val Arg Asp Arg Met Val Pro Pro Pro Pro Pro Pro
 545 550 555 560
 His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser Ser Ile Phe Leu His
 565 570 575
 Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile Ile Asn Tyr Thr Ile
 580 585 590
 Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser Leu Val Leu Tyr Leu
 595 600 605
 Gln Thr Ser Glu Thr His Met Leu Val Gln Gly Leu Glu Pro Asn Thr
 610 615 620
 Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp Gln Leu Ser Ser Pro
 625 630 635 640
 Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro Glu Ala Pro Ala Gly
 645 650 655
 Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu Asp Asp Thr Ala Leu
 660 665 670
 Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr Val Val Thr Arg Tyr
 675 680 685
 Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile Ala Gly Glu Trp Gln
 690 695 700
 Val Leu His Arg Glu Gly Ala Ile Thr Met Ala Leu Leu Glu Asn Leu
 705 710 715 720
 Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser Ala Ser Asn Glu Val
 725 730 735
 Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu Ala Val Leu Pro Lys
 740 745 750
 Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg Leu Asp Ser Ala Asp
 755 760 765
 Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp Gln Lys Ser Met Thr
 770 775 780
 Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr Cys Ile Leu Ile Cys
 785 790 795 800
 Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg Lys Ser Ser Ala Ser
 805 810 815
 Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro Arg Thr Ser Ala Ser
 820 825 830
 Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu Glu Gly Ala Val Gly
 835 840 845
 Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro Asn Ser Phe Ile Asp
 850 855 860
 Ala Lys Gly Gly Thr Asp Leu Ile Ile Asn Ser Tyr Gly Pro Ile Ile
 865 870 875 880
 Lys Asn Asn Ser Lys Lys Lys Trp Phe Phe Phe Gln Asp Ser Lys Lys
 885 890 895
 Ile Gln Val Glu Gln Pro Gln Arg Arg Phe Thr Pro Ala Val Cys Phe
 900 905 910
 Tyr Gln Pro Gly Thr Thr Val Leu Ile Ser Asp Glu Asp Ser Pro Ser
 915 920 925
 Ser Pro Gly Gln Thr Thr Ser Phe Ser Arg Pro Phe Gly Val Ala Ala
 930 935 940
 Asp Thr Glu His Ser Ala Asn Ser Glu Gly Ser His Glu Thr Gly Asp
 945 950 955 960

-continued

Ser Gly Arg Phe Ser His Glu Ser Asn Asp Glu Ile His Leu Ser Ser
 965 970 975

Val Ile Ser Thr Thr Pro Pro Asn Leu
 980 985

<210> SEQ ID NO 11
 <211> LENGTH: 2976
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 11

atgtctgaaa ataaacggat cgaggttctt tctaacggct ctttatacat cagtgagggtg 60
 gaaggcaggc gaggagagca gtccgatgaa ggattttatc agtgcttggc aatgaacaaa 120
 tatggagcca ttcttagtca aaaagctcat ctgccttat caactatttc tgcatttgaa 180
 gtccagccaa ttccactga ggtccacgaa ggtggagttg ctcgatttgc atgcaagatt 240
 tcatccccc cctctgcagt cataacatgg gagttcaatc ggacaactct acctatgact 300
 atggacagga taactgccct accaacagga gtattgcaga tctatgatgt cagccaaaagg 360
 gattctggaa attatcgttg tattgctgcc actgtagccc accgacgtaa aagtatggag 420
 gcctcgctaa ctgtgattcc agctaaggag tcaaaatcct tccacacacc arcaattata 480
 gcagggtccac agaacataac aacatctctt catcagactg tagttttgga atgcatggcc 540
 acaggaaaac ccaaaccaat catttcttgg agccgccttg atcacaatc cattgatgtc 600
 ttttaactac ggttacttgg aaatggtaat ctcatgatat ctgatgtcag gctacaacat 660
 gctggagtat atgtttgtcg ggccactacc cctggcacac gcaactttac agttgctatg 720
 gcaactttaa ctgtattagc tctccttca tttgttgaat ggccagaaag tttacaagg 780
 cctcgagctg gcaactgctg atttgtgtgt caggcagaag gaatccccct tcccaagatg 840
 tcatggttga aaatgggaag gaagatacat tcgaatggta gaattaaaat gtacaacagt 900
 aaatggtaa ttaaccagat tattcttgaa gatgatgcta tttatcagtg catggctgag 960
 aatagccaag gatctatttt atctagagcc agactgactg tagtgatgtc agaagacaga 1020
 cccagtgctc cctataatgt acatgtgaa accatgtcaa gctcagccat tcttttagcc 1080
 tgggagaggg cactttataa ttcagacaaa gtcattgcct attctgtaca ctacatgaaa 1140
 gcagaagggt taaataatga agagtatcaa gtagtcatcg gaaatgacac aactcattat 1200
 attattgatg acttagagcc tgccagcaat tatactttct acattgtagc atatatgcca 1260
 atgggagcca gccagatgtc tgaccatgtg acacagaata ctctagagga tgttccccctg 1320
 agacctcctg aaattagttt gacaagtcga agtcccactg atattctcat ctctgggtg 1380
 ccaatcccag ccaaatatcg gcggggccaa gtggtgctgt atcgcttgtc tttccgccta 1440
 agtactgaga attcaatcca agttctggag ctcccgggga ccacgcatga gtaccttttg 1500
 gaaggcctga aacctgacag tgtctacctg gttcggatta ctgctgccac cagagtgggg 1560
 ctgggagagt catcagatg gacttcacat aggacgccc aagctacaag cgtgaaagcc 1620
 cctaagtctc cagagttgca ttggagcct ctgaactgta ccaccatttc tgtgagggtg 1680
 cagcaagatg tagaggacac agctgctatt cagggtaca agctgtacta caaggaagaa 1740
 gggcagcagg agaatgggcc cattttcttg gataccaagg acctactcta tactctcagt 1800
 ggcttagacc ccagaagaaa atatcatgtg agactccttg cttacaacaa catagacgat 1860
 ggctatcagg cagatcagac tgtcagcact ccaggatgag tgtctgttcg tgatcgcatg 1920
 gtccctcctc caccaccacc ccaccatctc tatgcgaagg ctaacacctc atcttccatc 1980

-continued

```

ttctgcact ggaggagccc tgcattcacc gctgcacaaa tcattaacta caccatccgc 2040
tgtaatcctg ttggcctgca gaatgcttct ttggttctgt accttcaaac atcagaaact 2100
cacatgttgg ttcaaggctc agaaccacac accaaatagc aatttgccgt tcgattacat 2160
gtggatcagc ttccagtcct ttggagccct gtagtctacc attctactct tccagaagca 2220
ccagcaggcc caccagttgg agtaaaagt acattaatag aggatgacac tgccttggtt 2280
tcttggaac cccctgatgg ccagaaaaca gttgtgaccc gctatactat cttatatgca 2340
tctaggaagg cctggattgc aggagagtgg caggtcttac accgtgaagg ggcaataacc 2400
atggctttgc tagaaaactt ggtagcagga aatgtgtaca ttgtcaagat atctgcatcc 2460
aatgaggtgg gagaaggacc cttttcaaat tctgtggagc tggcagtact tccaaaggaa 2520
accttgaat caaatcagag gcccaagcgt ttagattctg ctgatgcaa agtttattca 2580
ggatattacc atctggacca aaaatcaatg actggcattg ctgtagggtg tggcatagcc 2640
ttgacctgca tcctcatctg tgtctcctc ttgatatacc gaagtaaagc caggaaatca 2700
tctgtctcca agacggcaca gaatggaact caacagttac ctctaccag tgcctcctta 2760
gctagtggaa atgaggtagg aaagaacctg gaaggagctg taggaaatga agaattctta 2820
atgccaatga tcatgccaaa cagcttcatt gatgcaaagg tactgagctg cggtgattgc 2880
tgcataagcc gttctccat tcctctctcc tgtgtgtgta aaatgtactt cccccaaat 2940
tgtatgttga atgtattata ccaatactct tattaa 2976

```

<210> SEQ ID NO 12
 <211> LENGTH: 991
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 12

```

Met Ser Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr
 1             5             10             15
Ile Ser Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe
          20             25             30
Tyr Gln Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys
          35             40             45
Ala His Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile
          50             55             60
Ser Thr Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile
          65             70             75             80
Ser Ser His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr
          85             90             95
Leu Pro Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu
          100            105            110
Gln Ile Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile
          115            120            125
Ala Ala Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr
          130            135            140
Val Ile Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile
          145            150            155            160
Ala Gly Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu
          165            170            175
Glu Cys Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg
          180            185            190

```

-continued

Leu Asp His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn
 195 200 205
 Gly Asn Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr
 210 215 220
 Val Cys Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met
 225 230 235 240
 Ala Thr Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu
 245 250 255
 Ser Leu Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala
 260 265 270
 Glu Gly Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys
 275 280 285
 Ile His Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile
 290 295 300
 Asn Gln Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu
 305 310 315 320
 Asn Ser Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met
 325 330 335
 Ser Glu Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met
 340 345 350
 Ser Ser Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser
 355 360 365
 Asp Lys Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu
 370 375 380
 Asn Asn Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr
 385 390 395 400
 Ile Ile Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val
 405 410 415
 Ala Tyr Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln
 420 425 430
 Asn Thr Leu Glu Asp Val Pro Leu Arg Pro Pro Glu Ile Ser Leu Thr
 435 440 445
 Ser Arg Ser Pro Thr Asp Ile Leu Ile Ser Trp Leu Pro Ile Pro Ala
 450 455 460
 Lys Tyr Arg Arg Gly Gln Val Val Leu Tyr Arg Leu Ser Phe Arg Leu
 465 470 475 480
 Ser Thr Glu Asn Ser Ile Gln Val Leu Glu Leu Pro Gly Thr Thr His
 485 490 495
 Glu Tyr Leu Leu Glu Gly Leu Lys Pro Asp Ser Val Tyr Leu Val Arg
 500 505 510
 Ile Thr Ala Ala Thr Arg Val Gly Leu Gly Glu Ser Ser Val Trp Thr
 515 520 525
 Ser His Arg Thr Pro Lys Ala Thr Ser Val Lys Ala Pro Lys Ser Pro
 530 535 540
 Glu Leu His Leu Glu Pro Leu Asn Cys Thr Thr Ile Ser Val Arg Trp
 545 550 555 560
 Gln Gln Asp Val Glu Asp Thr Ala Ala Ile Gln Gly Tyr Lys Leu Tyr
 565 570 575
 Tyr Lys Glu Glu Gly Gln Gln Glu Asn Gly Pro Ile Phe Leu Asp Thr
 580 585 590
 Lys Asp Leu Leu Tyr Thr Leu Ser Gly Leu Asp Pro Arg Arg Lys Tyr
 595 600 605
 His Val Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala

-continued

610	615	620
Asp Gln Thr Val Ser Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met 625	630	635 640
Val Pro Pro Pro Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr 645	650	655
Ser Ser Ser Ile Phe Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala 660	665	670
Gln Ile Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn 675	680	685
Ala Ser Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val 690	695	700
Gln Gly Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His 705	710	715 720
Val Asp Gln Leu Ser Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr 725	730	735
Leu Pro Glu Ala Pro Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu 740	745	750
Ile Glu Asp Asp Thr Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro 755	760	765
Glu Thr Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala 770	775	780
Trp Ile Ala Gly Glu Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr 785	790	795 800
Met Ala Leu Leu Glu Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys 805	810	815
Ile Ser Ala Ser Asn Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val 820	825	830
Glu Leu Ala Val Leu Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro 835	840	845
Lys Arg Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His 850	855	860
Leu Asp Gln Lys Ser Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala 865	870	875 880
Leu Thr Cys Ile Leu Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys 885	890	895
Ala Arg Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln 900	905	910
Leu Pro Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys 915	920	925
Asn Leu Glu Gly Ala Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile 930	935	940
Met Pro Asn Ser Phe Ile Asp Ala Lys Val Leu Ser Cys Gly Ile Cys 945	950	955 960
Cys Ile Ser Arg Ser Ser Ile Pro Pro Pro Cys Val Cys Lys Met Tyr 965	970	975
Phe Pro Gln Asn Cys Met Leu Asn Val Leu Tyr Gln Tyr Ser Tyr 980	985	990

<210> SEQ ID NO 13

<211> LENGTH: 909

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 13

-continued

atgtctgaaa ataaacggat cgaggttctt tctaacggct ctttatacat cagtgaagg	60
gaaggcaggc gaggagagca gtccgatgaa ggattttatc agtgcttgcc aatgaacaaa	120
tatggagcca ttcttagtca aaaagctcat cttgccttat caactatttc tgcatttgaa	180
gtccagccaa tttccactga ggtccacgaa ggtggagttg ctcgatttgc atgcaagatt	240
tcatcccccacc ctctgcagtc cataacatgg gagttcaatc ggacaactct acctatgact	300
atggacagga taactgcctt accaacagga gtattgcaga tctatgatgt cagccaaagg	360
gattctggaa attatcggtt tattgctgcc actgtagccc accgacgtaa aagtatggag	420
gcctcgctaa ctgtgatcc agctaaggag tcaaaatcct tccacacacc arcaattata	480
gcaggtccac agaacataac aacatctctt catcagactg tagttttgga atgcatggcc	540
acaggaaatc ccaaaccaat catttcttgg agccgccttg atcacaaatc cattgatgtc	600
tttaatactc gggtagcttg aaatggtaat ctcatgatat ctgatgtcag gctacaacat	660
gctggagtat atgtttgtcg ggccactacc cctggcacac gcaactttac agttgctatg	720
gcaactttaa ctgtattagc tctccttca tttgttgaat ggccagaaag tttaacaagg	780
cctcgagctg gcactgctg atttgtgtgt caggcagaag gaatcccctc tccaagatg	840
tcatggttga aaaatggaag gaagatacat tcgaatggta gaattaaaat gtacaacagg	900
tttaataaa	909

<210> SEQ ID NO 14
 <211> LENGTH: 302
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 14

Met Ser Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr	
1 5 10 15	
Ile Ser Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe	
20 25 30	
Tyr Gln Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys	
35 40 45	
Ala His Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile	
50 55 60	
Ser Thr Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile	
65 70 75 80	
Ser Ser His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr	
85 90 95	
Leu Pro Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu	
100 105 110	
Gln Ile Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile	
115 120 125	
Ala Ala Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr	
130 135 140	
Val Ile Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile	
145 150 155 160	
Ala Gly Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu	
165 170 175	
Glu Cys Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg	
180 185 190	
Leu Asp His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn	
195 200 205	

-continued

Gly	Asn	Leu	Met	Ile	Ser	Asp	Val	Arg	Leu	Gln	His	Ala	Gly	Val	Tyr
210						215						220			
Val	Cys	Arg	Ala	Thr	Thr	Pro	Gly	Thr	Arg	Asn	Phe	Thr	Val	Ala	Met
225					230					235					240
Ala	Thr	Leu	Thr	Val	Leu	Ala	Pro	Pro	Ser	Phe	Val	Glu	Trp	Pro	Glu
				245					250					255	
Ser	Leu	Thr	Arg	Pro	Arg	Ala	Gly	Thr	Ala	Arg	Phe	Val	Cys	Gln	Ala
			260					265					270		
Glu	Gly	Ile	Pro	Ser	Pro	Lys	Met	Ser	Trp	Leu	Lys	Asn	Gly	Arg	Lys
		275					280					285			
Ile	His	Ser	Asn	Gly	Arg	Ile	Lys	Met	Tyr	Asn	Arg	Phe	Lys		
	290					295					300				

<210> SEQ ID NO 15
 <211> LENGTH: 2481
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 15

atgtctgaaa ataaacggat cgagggttctt tctaacggct ctttatacat cagtggagtg	60
gaaggcagcg gaggagagca gtccgatgaa ggattttatc agtgcttggc aatgaacaaa	120
tatggagcca ttcttagtca aaaagctcat cttgccttat caactatttc tgcatttgaa	180
gtccagccaa ttccactga ggtccacgaa ggtggagttg ctcgatttgc atgcaagatt	240
tcattcccacc ctctgcagt cataacatgg gaggttcaatc ggacaactct acctatgact	300
atggacagga taactgcctt accaacagga gtattgcaga tctatgatgt cagccaaagg	360
gattctggaa attatcgttg tattgctgcc actgtagccc accgacgtaa aagtatggag	420
gcctcgctaa ctgtgattcc agctaaggag tcaaaatcct tccacacacc arcaattata	480
gcagggtccac agaacataac aacatctctt catcagactg tagttttgga atgcatggcc	540
acaggaaaatc ccaaaccaat catttcttgg agccgccttg atcacaaatc cattgatgtc	600
tttaatactc gggtaacttg aaatggtaat ctcatgatat ctgatgtcag gctacaacat	660
gctggagtat atgtttgtcg ggccactacc cctggcacac gcaactttac agttgctatg	720
gcaactttaa ctgtattagc tcctccttca ttgttgtaat ggccagaaag tttaacaagg	780
cctcgagctg gcactgctcg atttgtgtgt caggcagaag gaatccctc tcccaagatg	840
tcattggtga aaatgggaag gaagatacat tcgaatggta gaattaaaat gtacaacagt	900
aaattggtaa ttaaccagat tattcttgaa gatgatgcta tttatcagtg catggctgag	960
aatagccaag gatctatctt atctagagcc agactgactg tagtgatgtc agaagacaga	1020
cccagtgctc cctataatgt acatgctgaa accatgtcaa gctcagccat tcttttagcc	1080
tgggagaggg cactttataa ttacagacaaa gtcattgcct attctgtaca ctacatgaaa	1140
gcagaagggt taaataatga agagtatcaa gtagtcatcg gaaatgacac aactcattat	1200
attattgatg acttagagcc tgccagcaat tatactttct acattgtagc atatatgcca	1260
atgggagcca gccagatgtc tgaccatgtg acacagaata ctctagagga tgacccacga	1320
agaaaatata atgtgagact cctggcttac aacaacatag acgatggcta tcaggcagat	1380
cagactgtca gcactccagg atgcgtgtct gttcgtgacg gcatggctcc tcctccacca	1440
ccacccacc atctctatgc gaaggctaac acctcatctt ccatcttctt gcactggagg	1500
aggcctgcat tcacgcgtgc acaaatcatt aactacacca tccgctgtaa tcctgttggc	1560
ctgcagaatg cttcttttgt tctgtacctt caaacatcag aaactcacat gttggttcaa	1620

-continued

```

ggcttagaac caaacaccaa atacgaattt gccgttcgat tacatgtgga tcagctttcc 1680
agtccttgga gccctgtagt ctaccattct actcttcag aagcaccagc aggccaccaa 1740
gttggagtaa aagtacatt aatagaggat gacactgcc tggtttcttg gaaacccct 1800
gatggccag aaacagttgt gaccgctat actatcttat atgcatctag gaaggcctgg 1860
attgcaggag agtggcaggt ctacaccgt gaaggggcaa taaccatggc ttgctagaa 1920
aacttgtag caggaaatgt gtacattgtc aagatatctg catccaatga ggtgggagaa 1980
ggaccctttt caaattctgt ggagctggca gtacttcaa aggaacctc tgaatcaaat 2040
cagaggccca agcgtttaga ttctgctgat gccaaagttt attcaggata ttaccatctg 2100
gacaaaaaat caatgactgg cattgctgta ggtgttgga tagccttgac ctgcatctc 2160
atctgtgttc tcactctgat ataccgaagt aaagccagga aatcatctgc ttccaagacg 2220
gcacagaatg gaactcaaca gttacctgt accagtgcct cttagctag tggaaatgag 2280
gtaggaanga acctggaagg agctgttaga aatgaagaat ctttaatgcc aatgatcatg 2340
ccaaacagct tcattgatgc aaaggtactg agctgcggga ttgctgcat aagccgttct 2400
tccattctc ctcctgtgt gtgtaaaatg tacttcccc aaaattgtat gttgaatga 2460
ttataccaat actcttatta a
2481

```

<210> SEQ ID NO 16
 <211> LENGTH: 826
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 16

```

Met Ser Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr
 1         5         10        15
Ile Ser Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe
      20        25        30
Tyr Gln Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys
    35        40        45
Ala His Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile
    50        55        60
Ser Thr Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile
    65        70        75        80
Ser Ser His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr
      85        90        95
Leu Pro Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu
    100       105       110
Gln Ile Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile
    115       120       125
Ala Ala Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr
    130       135       140
Val Ile Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile
    145       150       155       160
Ala Gly Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu
    165       170       175
Glu Cys Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg
    180       185       190
Leu Asp His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn
    195       200       205
Gly Asn Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr

```

-continued

210	215	220
Val Cys Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met 225 230 235 240		
Ala Thr Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu 245 250 255		
Ser Leu Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala 260 265 270		
Glu Gly Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys 275 280 285		
Ile His Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile 290 295 300		
Asn Gln Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu 305 310 315 320		
Asn Ser Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met 325 330 335		
Ser Glu Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met 340 345 350		
Ser Ser Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser 355 360 365		
Asp Lys Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu 370 375 380		
Asn Asn Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr 385 390 395 400		
Ile Ile Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val 405 410 415		
Ala Tyr Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln 420 425 430		
Asn Thr Leu Glu Asp Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu 435 440 445		
Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln Thr Val Ser 450 455 460		
Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met Val Pro Pro Pro Pro 465 470 475 480		
Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser Ser Ile Phe 485 490 495		
Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile Ile Asn Tyr 500 505 510		
Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser Leu Val Leu 515 520 525		
Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val Gln Gly Leu Glu Pro 530 535 540		
Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp Gln Leu Ser 545 550 555 560		
Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro Glu Ala Pro 565 570 575		
Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu Asp Asp Thr 580 585 590		
Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr Val Val Thr 595 600 605		
Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile Ala Gly Glu 610 615 620		
Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr Met Ala Leu Leu Glu 625 630 635 640		

-continued

Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser Ala Ser Asn
 645 650 655
 Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu Ala Val Leu
 660 665 670
 Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg Leu Asp Ser
 675 680 685
 Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp Gln Lys Ser
 690 695 700
 Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr Cys Ile Leu
 705 710 715 720
 Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg Lys Ser Ser
 725 730 735
 Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro Arg Thr Ser
 740 745 750
 Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu Glu Gly Ala
 755 760 765
 Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro Asn Ser Phe
 770 775 780
 Ile Asp Ala Lys Val Leu Ser Cys Gly Ile Cys Cys Ile Ser Arg Ser
 785 790 795 800
 Ser Ile Pro Pro Pro Cys Val Cys Lys Met Tyr Phe Pro Gln Asn Cys
 805 810 815
 Met Leu Asn Val Leu Tyr Gln Tyr Ser Tyr
 820 825

<210> SEQ ID NO 17
 <211> LENGTH: 3219
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 17

atgtctgaaa ataaacggat cgagggtctt tctaacggct cttatacat cagtggagtg 60
 gaaggcaggc gaggagagca gtccgatgaa ggattttatc agtgcttggc atgaacaaa 120
 tatggagcca ttcttagtca aaaagctcat ctgacctat caactatttc tgcattgaa 180
 gtccagccaa tttccactga ggtccacgaa ggtggagtgt ctcgatttgc atgcaagatt 240
 tcatccccc ctcctgcagt cataacatgg gagttcaatc ggacaactct acctatgact 300
 atggacagga taactgccct accaacagga gtattgcaga tctatgatgt cagccaaaagg 360
 gattctggaa attatcgttg tattgctgcc actgtagccc accgacgtaa aagtatggag 420
 gcctcgctaa ctgtgattcc agctaaggag tcaaaatcct tccacacacc arcaattata 480
 gcagggtccac agaacataac aacatctctt catcagactg tagttttgga atgcatggcc 540
 acaggaaatc ccaaaccaat catttcttgg agcgccttgc atcacaaatc cattgatgtc 600
 tttaatactc gggtaacttg aaatggtaat ctcatgatat ctgatgtcag gctacaacat 660
 gctggagtat atgtttgtcg ggccactacc cctggcacac gcaactttac agttgctatg 720
 gcaactttaa ctgtattagc tcctccttca ttgttgaat ggccagaaag tttaacaagg 780
 cctcgagctg gcaactgctg atttgtgtgt caggcagaag gaatccctc tcccaagatg 840
 tcatgggtga aaaatggaag gaagatacat tcgaatggta gaattaaaat gtacaacagt 900
 aaattggtaa ttaaccagat tattcctgaa gatgatgcta tttatcagt catggctgag 960
 aatagccaag gatctatctt atctagagcc agactgactg tagtgatgtc agaagacaga 1020

-continued

cccagtgtc	cctataatgt	acatgtctgaa	accatgtcaa	gctcagccat	tcttttagcc	1080
tgggagaggc	cactttataa	ttcagacaaa	gtcattgcct	attctgtaca	ctacatgaaa	1140
gcagaagggt	taataaatga	agagtataca	gtagtcatcg	gaaatgacac	aactcattat	1200
attattgatg	acttagagcc	tgccagcaat	tatactttct	acattgtagc	atatatgcca	1260
atgggagcca	gccagatgtc	tgaccatgtg	acacagaata	ctctagagga	tgttcccctg	1320
agacctcctg	aaattagttt	gacaagtoga	agtcccactg	atattctcat	ctcctggctg	1380
ccaatcccag	ccaaatatcg	gcggggccaa	gtggtgctgt	atcgcttgtc	tttccgccta	1440
agtactgaga	attcaatcca	agttctggag	ctccggggga	ccacgcata	gtaccttttg	1500
gaaggcctga	aacctgacag	tgtctacctg	gttcggatta	ctgctgccac	cagagtgggg	1560
ctgggagagt	catcagtatg	gacttcacat	aggacgcccc	aagctacaag	cgtgaaagcc	1620
cctaagtctc	cagagttgca	tttgaggcct	ctgaactgta	ccaccatttc	tgtgagggtg	1680
cagcaagatg	tagaggacac	agctgctatt	cagggttaca	agctgtacta	caaggaagaa	1740
gggcagcagg	agaatgggac	cattttcttg	gataccaagg	acctactcta	tactctcagt	1800
ggcttagacc	ccagaagaaa	atatcatgtg	agactcctgg	cttacaacaa	catagacgat	1860
ggctatcagg	cagatcagac	tgtcagcact	ccaggatgcy	tgtctgttcg	tgatcgcatg	1920
gtccctctcc	caccaccacc	ccaccatctc	tatgcgaagg	ctaacacctc	atcttccatc	1980
ttcctgcact	ggaggaggcc	tgcatccacc	gctgcacaaa	tcattaaacta	caccatccgc	2040
tgtaatcctg	ttggcctgca	gaatgcttct	ttggttctgt	accttcaaac	atcagaaact	2100
cacatgttgg	ttcaaggctc	agaaccacac	accaaatagc	aatttgccgt	tcgattacat	2160
gtggatcagc	tttcagtc	ttggagccct	gtagtctacc	attctactct	tccagaagca	2220
ccagcagccc	caccagttgg	agtaaaagt	acattaatag	aggatgacac	tgccctgggt	2280
tcttggaac	cccctgatgg	cccagaaaca	gttgtgaccc	gctatactat	cttatatgca	2340
tctaggaagg	cctggattgc	aggagagtgg	caggctttac	accgtgaagg	ggcaataacc	2400
atggctttgc	tagaaaactt	ggtagcagga	aatgtgtaca	ttgtcaagat	atctgcatcc	2460
aatgaggtgg	gagaaggacc	cttttcaaat	tctgtggagc	tggcagtact	tccaaaggaa	2520
acctctgaat	caaatacag	gcccagcgt	ttagattctg	ctgatgcaa	agtttatcca	2580
ggatattacc	atctggacca	aaaatcaatg	actggcattg	ctgtagggtg	tggcatagcc	2640
ttgacctgca	tcctcatctg	tgttctcatc	ttgatatacc	gaagtaagc	caggaaatca	2700
tctgcttcca	agacggcaca	gaatggaact	caacagttac	ctcgtaccag	tgccctotta	2760
gctagtggaa	atgaggtagg	aaagaacctg	gaaggagctg	taggaaatga	agaatcttta	2820
atgccaatga	tcatgccaaa	cagcttcatt	gatgcaagg	gaggaactga	cctgataatt	2880
aatagctatg	gtcctataat	taaaaacac	tctaagaaaa	agtgtttttt	tttccaagac	2940
tcaaagaaga	tacaagttga	gcagcctcaa	agaagattta	ctccagcggg	ctgcttttac	3000
cagccaggca	ccactgtatt	aatcagtgat	gaagactccc	ctagctcccc	aggcagaca	3060
accagcttct	caagaccctt	tggtgttgca	gctgatacag	aacattcagc	aaatagtga	3120
ggcagccatg	agactgggga	ttctggggcg	ttttctcatg	agtccaacga	tgagatacat	3180
ctgtcctcag	ttataagtac	cacacccccc	aacctctga			3219

<210> SEQ ID NO 18

<211> LENGTH: 1072

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 18

Met Ser Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr
 1 5 10 15
 Ile Ser Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe
 20 25 30
 Tyr Gln Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys
 35 40 45
 Ala His Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile
 50 55 60
 Ser Thr Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile
 65 70 75 80
 Ser Ser His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr
 85 90 95
 Leu Pro Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu
 100 105 110
 Gln Ile Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile
 115 120 125
 Ala Ala Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr
 130 135 140
 Val Ile Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile
 145 150 155 160
 Ala Gly Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu
 165 170 175
 Glu Cys Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg
 180 185 190
 Leu Asp His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn
 195 200 205
 Gly Asn Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr
 210 215 220
 Val Cys Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met
 225 230 235 240
 Ala Thr Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu
 245 250 255
 Ser Leu Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala
 260 265 270
 Glu Gly Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys
 275 280 285
 Ile His Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile
 290 295 300
 Asn Gln Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu
 305 310 315 320
 Asn Ser Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met
 325 330 335
 Ser Glu Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met
 340 345 350
 Ser Ser Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser
 355 360 365
 Asp Lys Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu
 370 375 380
 Asn Asn Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr
 385 390 395 400
 Ile Ile Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val

-continued

405	410	415
Ala Tyr Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln 420	425	430
Asn Thr Leu Glu Asp Val Pro Leu Arg Pro Pro Glu Ile Ser Leu Thr 435	440	445
Ser Arg Ser Pro Thr Asp Ile Leu Ile Ser Trp Leu Pro Ile Pro Ala 450	455	460
Lys Tyr Arg Arg Gly Gln Val Val Leu Tyr Arg Leu Ser Phe Arg Leu 465	470	475
Ser Thr Glu Asn Ser Ile Gln Val Leu Glu Leu Pro Gly Thr Thr His 485	490	495
Glu Tyr Leu Leu Glu Gly Leu Lys Pro Asp Ser Val Tyr Leu Val Arg 500	505	510
Ile Thr Ala Ala Thr Arg Val Gly Leu Gly Glu Ser Ser Val Trp Thr 515	520	525
Ser His Arg Thr Pro Lys Ala Thr Ser Val Lys Ala Pro Lys Ser Pro 530	535	540
Glu Leu His Leu Glu Pro Leu Asn Cys Thr Thr Ile Ser Val Arg Trp 545	550	555
Gln Gln Asp Val Glu Asp Thr Ala Ala Ile Gln Gly Tyr Lys Leu Tyr 565	570	575
Tyr Lys Glu Glu Gly Gln Gln Glu Asn Gly Pro Ile Phe Leu Asp Thr 580	585	590
Lys Asp Leu Leu Tyr Thr Leu Ser Gly Leu Asp Pro Arg Arg Lys Tyr 595	600	605
His Val Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala 610	615	620
Asp Gln Thr Val Ser Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met 625	630	635
Val Pro Pro Pro Pro Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr 645	650	655
Ser Ser Ser Ile Phe Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala 660	665	670
Gln Ile Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn 675	680	685
Ala Ser Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val 690	695	700
Gln Gly Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His 705	710	715
Val Asp Gln Leu Ser Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr 725	730	735
Leu Pro Glu Ala Pro Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu 740	745	750
Ile Glu Asp Asp Thr Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro 755	760	765
Glu Thr Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala 770	775	780
Trp Ile Ala Gly Glu Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr 785	790	795
Met Ala Leu Leu Glu Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys 805	810	815
Ile Ser Ala Ser Asn Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val 820	825	830

-continued

Glu Leu Ala Val Leu Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro
 835 840 845
 Lys Arg Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His
 850 855 860
 Leu Asp Gln Lys Ser Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala
 865 870 875 880
 Leu Thr Cys Ile Leu Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys
 885 890 895
 Ala Arg Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln
 900 905 910
 Leu Pro Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys
 915 920 925
 Asn Leu Glu Gly Ala Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile
 930 935 940
 Met Pro Asn Ser Phe Ile Asp Ala Lys Gly Gly Thr Asp Leu Ile Ile
 945 950 955 960
 Asn Ser Tyr Gly Pro Ile Ile Lys Asn Asn Ser Lys Lys Lys Trp Phe
 965 970 975
 Phe Phe Gln Asp Ser Lys Lys Ile Gln Val Glu Gln Pro Gln Arg Arg
 980 985 990
 Phe Thr Pro Ala Val Cys Phe Tyr Gln Pro Gly Thr Thr Val Leu Ile
 995 1000 1005
 Ser Asp Glu Asp Ser Pro Ser Ser Pro Gly Gln Thr Thr Ser Phe Ser
 1010 1015 1020
 Arg Pro Phe Gly Val Ala Ala Asp Thr Glu His Ser Ala Asn Ser Glu
 1025 1030 1035 1040
 Gly Ser His Glu Thr Gly Asp Ser Gly Arg Phe Ser His Glu Ser Asn
 1045 1050 1055
 Asp Glu Ile His Leu Ser Ser Val Ile Ser Thr Thr Pro Pro Asn Leu
 1060 1065 1070

<210> SEQ ID NO 19
 <211> LENGTH: 2724
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 19

atgtctgaaa ataaacggat cgaggttctt tctaacggct ctttatacat cagttaggtg 60
 gaaggcaggc gaggagagca gtccgatgaa ggattttatc agtgcttggc aatgaacaaa 120
 tatggagcca ttcttagtca aaaagctcat ctgaccttat caactatttc tgcatttgaa 180
 gtccagccaa ttccactga ggtccacgaa ggtggagttg ctcgatttgc atgcaagatt 240
 tcatcccacc ctctgcagt cataacatgg gagttcaatc ggacaactct acctatgact 300
 atggacagga taactgccct accaacagga gtattgcaga tctatgatgt cagccaaagg 360
 gattctggaa attatcgttg tattgctgcc actgtagccc accgacgtaa aagtatggag 420
 gctcgctaa ctgtgattcc agctaaggag tcaaaatcct tccacacacc arcaattata 480
 gcagggtccc agaacataac aacatctctt catcagactg tagttttgga atgcatggcc 540
 acaggaaato ccaaaccaat catttcttgg agccgccttg atcacaaatc cattgatgtc 600
 tttatactc gggtagtttg aaatggtaat ctcatgatat ctgatgtcag gctacaacat 660
 gctggagtat atgtttgtcg ggccactacc cctggcacac gcaactttac agttgctatg 720
 gcaactttaa ctgtattagc tcctccttca ttgttgaat ggccagaaag ttaacaagg 780

-continued

```

cctcgagctg gcaactgctcg atttgtgtgt caggcagaag gaatccccctc tcccaagatg 840
tcattggtga aaatggaag gaagatacat tcgaatgga gaattaaaat gtacaacagt 900
aaattggtaa ttaaccagat tattcctgaa gatgatgcta tttatcagt catggctgag 960
aatagccaag gatctatatt atctagagcc agactgactg tagtgatgac agaagacaga 1020
cccagtgtc cctataatgt acatgctgaa accatgtcaa gctcagccat tcttttagcc 1080
tgaggagagg cactttataa ttcagacaaa gtcattgcct attctgtaca ctacatgaaa 1140
gcagaagggt taaataatga agagtatcaa gtagtcacg gaaatgacac aactcattat 1200
attattgatg acttagagcc tgccagcaat tatactttct acattgtagc atatatgcca 1260
atgggagcca gccagatgtc tgaccatgtg acacagaata ctctagagga tgaccccgaga 1320
agaaaatata atgtgagact cctggcttac aacaacatag acgatggcta tcaggcagat 1380
cagactgtca gcactccagg atgcgtgtct gtctgtgac gcatggctcc tctccacca 1440
ccaacccacc atctctatgc gaaggctaac acotcatctt ccatcttctt gcactggagg 1500
aggcctgcat tcaccgtgc acaaatcatt aactacacca tccgtgttaa tctgttggc 1560
ctgcagaatg cttcttgggt tctgtacctt caaacatcag aaactcacat gttggttcaa 1620
ggctctagaac caaacaccaa atacgaattt gccgttcgat tacatgtgga tcagctttcc 1680
agtccttgga gccctgtagt ctaccattct actcttcag aagcaccagc aggccacca 1740
gttgagtaga aagtgcatt aatagaggat gacactgcc tggtttcttg gaaacccct 1800
gatggcccag aaacagttgt gacccgtat actatcttat atgcactag gaaggcctgg 1860
attgcaggag agtggcagg cttacaccgt gaaggggcaa taacatggc tttgctagaa 1920
aacttggtag caggaatagt gtacattgtc aagatatctg catccaatga ggtgggagaa 1980
ggaccctttt caaatctgt ggagctggca gtacttcaa aggaacctc tgaatcaaat 2040
cagaggccca agcgtttaga ttctgtgat gccaaagttt attcaggata ttaccatctg 2100
gacaaaaaat caatgactgg cattgtgta ggtgttgga tagccttgac ctgcacctc 2160
atctgtgttc tcactctgat ataccgaagt aaagccagga aatcatctgc ttccaagacg 2220
gcacagaatg gaactcaaca gttacctgt accagtgcct ccttagctag tggaatgag 2280
gtaggaaaga acctggaagg agctgtagga aatgaagaat ctttaatgcc aatgatcatg 2340
ccaaacagct tcattgatgc aaaggagga actgacctga taattaatag ctatggctct 2400
ataattaaaa acaactctaa gaaaaagtgg ttttttttcc aagactcaa gaagatacaa 2460
gttgagcagc ctcaagaag atttactcca gcgtctgct tttaccagcc aggcaccact 2520
gtattaatca gtgatgaaga ctcccctagc tcccaggtc agaacaaccg cttctcaaga 2580
cccttggtg ttgcagctga tacagaacat tcagcaata gtgaaggcag ccatgagact 2640
ggggattctg ggcggtttct tcattgagtc aacgatgaga tacatctgtc ctcaattata 2700
agtaccacac ccccaacct ctga 2724

```

<210> SEQ ID NO 20

<211> LENGTH: 907

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 20

```

Met Ser Glu Asn Lys Arg Ile Glu Val Leu Ser Asn Gly Ser Leu Tyr
 1           5           10          15

```

```

Ile Ser Glu Val Glu Gly Arg Arg Gly Glu Gln Ser Asp Glu Gly Phe

```


-continued

20	25	30
Tyr Gln Cys Leu Ala Met Asn Lys Tyr Gly Ala Ile Leu Ser Gln Lys 35 40 45		
Ala His Leu Ala Leu Ser Thr Ile Ser Ala Phe Glu Val Gln Pro Ile 50 55 60		
Ser Thr Glu Val His Glu Gly Gly Val Ala Arg Phe Ala Cys Lys Ile 65 70 75 80		
Ser Ser His Pro Pro Ala Val Ile Thr Trp Glu Phe Asn Arg Thr Thr 85 90 95		
Leu Pro Met Thr Met Asp Arg Ile Thr Ala Leu Pro Thr Gly Val Leu 100 105 110		
Gln Ile Tyr Asp Val Ser Gln Arg Asp Ser Gly Asn Tyr Arg Cys Ile 115 120 125		
Ala Ala Thr Val Ala His Arg Arg Lys Ser Met Glu Ala Ser Leu Thr 130 135 140		
Val Ile Pro Ala Lys Glu Ser Lys Ser Phe His Thr Pro Thr Ile Ile 145 150 155 160		
Ala Gly Pro Gln Asn Ile Thr Thr Ser Leu His Gln Thr Val Val Leu 165 170 175		
Glu Cys Met Ala Thr Gly Asn Pro Lys Pro Ile Ile Ser Trp Ser Arg 180 185 190		
Leu Asp His Lys Ser Ile Asp Val Phe Asn Thr Arg Val Leu Gly Asn 195 200 205		
Gly Asn Leu Met Ile Ser Asp Val Arg Leu Gln His Ala Gly Val Tyr 210 215 220		
Val Cys Arg Ala Thr Thr Pro Gly Thr Arg Asn Phe Thr Val Ala Met 225 230 235 240		
Ala Thr Leu Thr Val Leu Ala Pro Pro Ser Phe Val Glu Trp Pro Glu 245 250 255		
Ser Leu Thr Arg Pro Arg Ala Gly Thr Ala Arg Phe Val Cys Gln Ala 260 265 270		
Glu Gly Ile Pro Ser Pro Lys Met Ser Trp Leu Lys Asn Gly Arg Lys 275 280 285		
Ile His Ser Asn Gly Arg Ile Lys Met Tyr Asn Ser Lys Leu Val Ile 290 295 300		
Asn Gln Ile Ile Pro Glu Asp Asp Ala Ile Tyr Gln Cys Met Ala Glu 305 310 315 320		
Asn Ser Gln Gly Ser Ile Leu Ser Arg Ala Arg Leu Thr Val Val Met 325 330 335		
Ser Glu Asp Arg Pro Ser Ala Pro Tyr Asn Val His Ala Glu Thr Met 340 345 350		
Ser Ser Ser Ala Ile Leu Leu Ala Trp Glu Arg Pro Leu Tyr Asn Ser 355 360 365		
Asp Lys Val Ile Ala Tyr Ser Val His Tyr Met Lys Ala Glu Gly Leu 370 375 380		
Asn Asn Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His Tyr 385 390 395 400		
Ile Ile Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile Val 405 410 415		
Ala Tyr Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr Gln 420 425 430		
Asn Thr Leu Glu Asp Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu 435 440 445		

-continued

Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln Thr Val Ser
 450 455 460
 Thr Pro Gly Cys Val Ser Val Arg Asp Arg Met Val Pro Pro Pro Pro
 465 470 475 480
 Pro Pro His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser Ser Ile Phe
 485 490 495
 Leu His Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile Ile Asn Tyr
 500 505 510
 Thr Ile Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser Leu Val Leu
 515 520 525
 Tyr Leu Gln Thr Ser Glu Thr His Met Leu Val Gln Gly Leu Glu Pro
 530 535 540
 Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp Gln Leu Ser
 545 550 555 560
 Ser Pro Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro Glu Ala Pro
 565 570 575
 Ala Gly Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu Asp Asp Thr
 580 585 590
 Ala Leu Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr Val Val Thr
 595 600 605
 Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile Ala Gly Glu
 610 615 620
 Trp Gln Val Leu His Arg Glu Gly Ala Ile Thr Met Ala Leu Leu Glu
 625 630 635 640
 Asn Leu Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser Ala Ser Asn
 645 650 655
 Glu Val Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu Ala Val Leu
 660 665 670
 Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg Leu Asp Ser
 675 680 685
 Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp Gln Lys Ser
 690 695 700
 Met Thr Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr Cys Ile Leu
 705 710 715 720
 Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg Lys Ser Ser
 725 730 735
 Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro Arg Thr Ser
 740 745 750
 Ala Ser Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu Glu Gly Ala
 755 760 765
 Val Gly Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro Asn Ser Phe
 770 775 780
 Ile Asp Ala Lys Gly Gly Thr Asp Leu Ile Ile Asn Ser Tyr Gly Pro
 785 790 795 800
 Ile Ile Lys Asn Asn Ser Lys Lys Lys Trp Phe Phe Phe Gln Asp Ser
 805 810 815
 Lys Lys Ile Gln Val Glu Gln Pro Gln Arg Arg Phe Thr Pro Ala Val
 820 825 830
 Cys Phe Tyr Gln Pro Gly Thr Thr Val Leu Ile Ser Asp Glu Asp Ser
 835 840 845
 Pro Ser Ser Pro Gly Gln Thr Thr Ser Phe Ser Arg Pro Phe Gly Val
 850 855 860

-continued

Ala	Ala	Asp	Thr	Glu	His	Ser	Ala	Asn	Ser	Glu	Gly	Ser	His	Glu	Thr
865					870					875				880	
Gly	Asp	Ser	Gly	Arg	Phe	Ser	His	Glu	Ser	Asn	Asp	Glu	Ile	His	Leu
			885						890				895		
Ser	Ser	Val	Ile	Ser	Thr	Thr	Pro	Pro	Asn	Leu					
			900						905						

<210> SEQ ID NO 21
 <211> LENGTH: 2139
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 21

```

atgtcatggt tgaaaaatgg aaggaagata cattcgaatg gtagaattaa aatgtacaac      60
agtaaattgg taattaacca gattattcct gaagatgatg ctatttatca gtgcatggct      120
gagaatagcc aaggatctat tttatctaga gccagactga ctgtagtgat gtcagaagac      180
agaccacgtg ctccctataa tgtacatgct gaaacacatgt caagctcagc cattctttta      240
gcctgggaga ggccacttta taattcagac aaagtcattg cctattctgt acactacatg      300
aaagcagaag gtttaataaa tgaagagtat caagtagtca tcggaaatga cacaactcat      360
tatattattg atgacttaga gcctgccagc aattatactt tctacattgt agcatatatg      420
ccaatgggag ccagccagat gtctgacct gtgacacaga atactctaga ggatgttccc      480
ctgagaccct ctgaaattag ttgacaagt cgaagtccca ctgatattct catctcctgg      540
ctgccaatcc cagccaaata tcggcggggc caagtgggtg tgtatcgctt gtctttccgc      600
ctaagtactg agaattcaat ccaagttctg gagctcccg ggaccacgca tgagtacctt      660
ttggaagccc tgaaacctga cagtgtctac ctggttcgga ttactgtctg caccagagtg      720
gggctgggag agtcatcagt atggacttca cataggacgc ccaaagctac aagcgtgaaa      780
gcccctaagt ctccagagtt gcatttggag cctctgaact gtaccacat ttctgtgagg      840
tggcagcaag atgtagagga cacagctgct attcagggct acaagctgta ctacaaggaa      900
gaagggcagc aggagaatgg gccattttc ttggatacca aggacctact ctatactctc      960
agtggcttag accccagaag aaaatatcat gtgagactcc tggettacaa caacatagac     1020
gatggctatc aggcagatca gactgtcagc actccaggat gcgtgtctgt tcgtgatcgc     1080
atggtccctc ctccaccacc accccaccat ctctatgcga aggctaacac ctcatcttcc     1140
atcttcctgc actggaggag gcctgcattc accgctgcac aaatcattaa ctacaccatc     1200
cgctgtaatc ctgttgccct gcagaatgct tctttgggtc tgtaccttca aacatcagaa     1260
actcacatgt tggttcaagg tctagaacca aacaccaaata acgaatttgc cgttcgatta     1320
catgtggatc agctttccag tccttggagc cctgtagtct accattctac tcttccagaa     1380
gcaccagcag gccaccacgt tggagtaaaa gtgacattaa tagaggatga cactgccctg     1440
gtttcttggg aaccctctga tggcccagaa acagtgtgta cccgtatac tatcttatat     1500
gcatctagga aggcctggat tgcaggagag tggcagggtc tacaccgtga aggggcaata     1560
accatggctt tgctagaaaa cttggtagca ggaaatgtgt acattgtcaa gatattctgca     1620
tccaatgagg tgggagaagg acccttttca aattctgttg agctggcagt acttccaang     1680
gaaacctctg aatcaaatca gaggcccaag cgttttagatt ctgctgatgc caaagtttat     1740
tcaggatatt accatctgga ccaaaaatca atgactggca ttgctgtagg tgttggcata     1800
gccttgacct gcatectcat ctgtgttctc atcttgatat accgaagtaa agccaggaaa     1860
  
```

-continued

tcattctgctt ccaagacggc acagaatgga actcaacagt tacctcgtac cagtcgctcc 1920
 ttagctagtg gaaatgaggt aggaagaac ctggaaggag ctgtaggaaa tgaagaatct 1980
 ttaatgcaa tgatcatgcc aaacagcttc attgatgcaa aggtactgag ctgcgggatt 2040
 tgctgcataa gccgttcttc cattctcct ccctgtgtgt gtaaatgta cttcccccac 2100
 aattgtatgt tgaatgtatt ataccaatac tcttattaa 2139

<210> SEQ ID NO 22
 <211> LENGTH: 712
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 22

Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His Ser Asn Gly Arg Ile
 1 5 10 15
 Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln Ile Ile Pro Glu Asp
 20 25 30
 Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser Gln Gly Ser Ile Leu
 35 40 45
 Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu Asp Arg Pro Ser Ala
 50 55 60
 Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser Ser Ala Ile Leu Leu
 65 70 75 80
 Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys Val Ile Ala Tyr Ser
 85 90 95
 Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn Glu Glu Tyr Gln Val
 100 105 110
 Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile Asp Asp Leu Glu Pro
 115 120 125
 Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr Met Pro Met Gly Ala
 130 135 140
 Ser Gln Met Ser Asp His Val Thr Gln Asn Thr Leu Glu Asp Val Pro
 145 150 155 160
 Leu Arg Pro Pro Glu Ile Ser Leu Thr Ser Arg Ser Pro Thr Asp Ile
 165 170 175
 Leu Ile Ser Trp Leu Pro Ile Pro Ala Lys Tyr Arg Arg Gly Gln Val
 180 185 190
 Val Leu Tyr Arg Leu Ser Phe Arg Leu Ser Thr Glu Asn Ser Ile Gln
 195 200 205
 Val Leu Glu Leu Pro Gly Thr Thr His Glu Tyr Leu Leu Glu Gly Leu
 210 215 220
 Lys Pro Asp Ser Val Tyr Leu Val Arg Ile Thr Ala Ala Thr Arg Val
 225 230 235 240
 Gly Leu Gly Glu Ser Ser Val Trp Thr Ser His Arg Thr Pro Lys Ala
 245 250 255
 Thr Ser Val Lys Ala Pro Lys Ser Pro Glu Leu His Leu Glu Pro Leu
 260 265 270
 Asn Cys Thr Thr Ile Ser Val Arg Trp Gln Gln Asp Val Glu Asp Thr
 275 280 285
 Ala Ala Ile Gln Gly Tyr Lys Leu Tyr Tyr Lys Glu Glu Gly Gln Gln
 290 295 300
 Glu Asn Gly Pro Ile Phe Leu Asp Thr Lys Asp Leu Leu Tyr Thr Leu
 305 310 315 320
 Ser Gly Leu Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr

-continued

325				330				335							
Asn	Asn	Ile	Asp	Asp	Gly	Tyr	Gln	Ala	Asp	Gln	Thr	Val	Ser	Thr	Pro
340				345				350							
Gly	Cys	Val	Ser	Val	Arg	Asp	Arg	Met	Val	Pro	Pro	Pro	Pro	Pro	Pro
355				360				365							
His	His	Leu	Tyr	Ala	Lys	Ala	Asn	Thr	Ser	Ser	Ser	Ile	Phe	Leu	His
370				375				380							
Trp	Arg	Arg	Pro	Ala	Phe	Thr	Ala	Ala	Gln	Ile	Ile	Asn	Tyr	Thr	Ile
385				390				395				400			
Arg	Cys	Asn	Pro	Val	Gly	Leu	Gln	Asn	Ala	Ser	Leu	Val	Leu	Tyr	Leu
405				410				415							
Gln	Thr	Ser	Glu	Thr	His	Met	Leu	Val	Gln	Gly	Leu	Glu	Pro	Asn	Thr
420				425				430							
Lys	Tyr	Glu	Phe	Ala	Val	Arg	Leu	His	Val	Asp	Gln	Leu	Ser	Ser	Pro
435				440				445							
Trp	Ser	Pro	Val	Val	Tyr	His	Ser	Thr	Leu	Pro	Glu	Ala	Pro	Ala	Gly
450				455				460							
Pro	Pro	Val	Gly	Val	Lys	Val	Thr	Leu	Ile	Glu	Asp	Asp	Thr	Ala	Leu
465				470				475				480			
Val	Ser	Trp	Lys	Pro	Pro	Asp	Gly	Pro	Glu	Thr	Val	Val	Thr	Arg	Tyr
485				490				495							
Thr	Ile	Leu	Tyr	Ala	Ser	Arg	Lys	Ala	Trp	Ile	Ala	Gly	Glu	Trp	Gln
500				505				510							
Val	Leu	His	Arg	Glu	Gly	Ala	Ile	Thr	Met	Ala	Leu	Leu	Glu	Asn	Leu
515				520				525							
Val	Ala	Gly	Asn	Val	Tyr	Ile	Val	Lys	Ile	Ser	Ala	Ser	Asn	Glu	Val
530				535				540							
Gly	Glu	Gly	Pro	Phe	Ser	Asn	Ser	Val	Glu	Leu	Ala	Val	Leu	Pro	Lys
545				550				555				560			
Glu	Thr	Ser	Glu	Ser	Asn	Gln	Arg	Pro	Lys	Arg	Leu	Asp	Ser	Ala	Asp
565				570				575							
Ala	Lys	Val	Tyr	Ser	Gly	Tyr	Tyr	His	Leu	Asp	Gln	Lys	Ser	Met	Thr
580				585				590							
Gly	Ile	Ala	Val	Gly	Val	Gly	Ile	Ala	Leu	Thr	Cys	Ile	Leu	Ile	Cys
595				600				605							
Val	Leu	Ile	Leu	Ile	Tyr	Arg	Ser	Lys	Ala	Arg	Lys	Ser	Ser	Ala	Ser
610				615				620							
Lys	Thr	Ala	Gln	Asn	Gly	Thr	Gln	Gln	Leu	Pro	Arg	Thr	Ser	Ala	Ser
625				630				635				640			
Leu	Ala	Ser	Gly	Asn	Glu	Val	Gly	Lys	Asn	Leu	Glu	Gly	Ala	Val	Gly
645				650				655							
Asn	Glu	Glu	Ser	Leu	Met	Pro	Met	Ile	Met	Pro	Asn	Ser	Phe	Ile	Asp
660				665				670							
Ala	Lys	Val	Leu	Ser	Cys	Gly	Ile	Cys	Cys	Ile	Ser	Arg	Ser	Ser	Ile
675				680				685							
Pro	Pro	Pro	Cys	Val	Cys	Lys	Met	Tyr	Phe	Pro	Gln	Asn	Cys	Met	Leu
690				695				700							
Asn	Val	Leu	Tyr	Gln	Tyr	Ser	Tyr								
705				710											

<210> SEQ ID NO 23

<211> LENGTH: 1875

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 23

```

atggaaggaa gatacattcg aatggtagaa ttaaaatgta caacagggtt aaataatgaa    60
gagtatcaag tagtcatcgg aaatgacaca actcattata ttattgatga cttagagcct    120
gccagcaatt atactttcta cattgtagca tatatgccaa tgggagccag ccagatgtct    180
gacctgtga cacagaatac tctagaggat gttccctga gacctcctga aattagtttg    240
acaagtcgaa gtcccactga tattctcatc tcttggtgc caatcccagc caaatatcgg    300
cggggccaag tgggtgtgta tcgcttgtct ttccgcctaa gtactgagaa ttcaatccaa    360
gttctggagc tcccggggac cagcatgag taccttttgg aaggcctgaa acctgacagt    420
gtctacctgg ttccgattac tgctgccacc agagtggggc tgggagagtc atcagtatgg    480
acttcacata ggacgcccaa agctacaagc gtgaaagccc ctaagtctcc agagttgcat    540
ttggagcctc tgaactgtac caccatttct gtgaggtggc agcaagatgt agaggacaca    600
gctgtattc agggctacaa gctgtactac aaggaagaag ggcagcagga gaatggggcc    660
atcttcttgg ataccaagga cctactctat actctcagtg gcttagaccc cagaagaaaa    720
tatcatgtga gactcctggc ttacaacaac atagacgatg gctatcaggc agatcagact    780
gtcagcactc caggatgcgt gtctgttcgt gatcgcatgg tccctcctcc accaccacc    840
caccatctct atgcgaaggc taacacotca tcttccatct tctgcactg gaggaggcct    900
gcattcaccg ctgcacaaat cattaactac accatccgct gtaatcctgt tggcctgcag    960
aatgcttctt tggttctgta ccttcaaaca tcagaaactc acatgttggt tcaaggtcta   1020
gaaccaaaca ccaaatacga atttgccgtt cgattacatg tggatcagct ttccagtcct   1080
tggagccctg tagtctacca ttctactctt ccagaagcac cagcaggccc accagttgga   1140
gtaaaagtga cattaataga ggatgacact gccctgggtt cttggaaacc cctgatggc   1200
ccagaacacg ttgtgacctg ctatactatc ttatagcat ctaggaaagg ctggattgca   1260
ggagagtggc aggtcttaca ccgtgaaggg gcaataacca tggctttgct agaaaacttg   1320
gtagcaggaa atgtgtacat tgtcaagata tctgcaccca atgaggtggg agaaggaccc   1380
ttttcaaatt ctgtggagct ggcagtactt ccaaggaaaa cctctgaatc aaatcagagg   1440
cccaagcgtt tagattctgc tgatgccaaa gtttattcag gatattacca tctggaccaaa   1500
aaatcaatga ctggcattgc tgtaggtggt ggcatagcct tgacctgcat cctcatctgt   1560
gttctcatct tgatataccg aagtaaagcc aggaaatcat ctgcttccaa gacggcacag   1620
aatggaactc aacagttacc tcgtaccagt gcctccttag ctagtggaaa tgaggtagga   1680
aagaacctgg aaggagctgt aggaatgaa gaatctttaa tgccaatgat catgccaaac   1740
agcttcattg atgcaaaggt actgagctgc gggatttgct gcataagccg ttcttccatt   1800
cctcctccct gtgtgtgtaa aatgtacttc ccccaaaatt gtatgttgaa tgtattatac   1860
caatactctt attaa                                         1875

```

<210> SEQ ID NO 24

<211> LENGTH: 624

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 24

```

Met Glu Gly Arg Tyr Ile Arg Met Val Glu Leu Lys Cys Thr Thr Gly
 1           5           10           15
Leu Asn Asn Glu Glu Tyr Gln Val Val Ile Gly Asn Asp Thr Thr His

```

-continued

20	25	30
Tyr Ile Ile Asp Asp Leu Glu Pro Ala Ser Asn Tyr Thr Phe Tyr Ile 35 40 45		
Val Ala Tyr Met Pro Met Gly Ala Ser Gln Met Ser Asp His Val Thr 50 55 60		
Gln Asn Thr Leu Glu Asp Val Pro Leu Arg Pro Pro Glu Ile Ser Leu 65 70 75 80		
Thr Ser Arg Ser Pro Thr Asp Ile Leu Ile Ser Trp Leu Pro Ile Pro 85 90 95		
Ala Lys Tyr Arg Arg Gly Gln Val Val Leu Tyr Arg Leu Ser Phe Arg 100 105 110		
Leu Ser Thr Glu Asn Ser Ile Gln Val Leu Glu Leu Pro Gly Thr Thr 115 120 125		
His Glu Tyr Leu Leu Glu Gly Leu Lys Pro Asp Ser Val Tyr Leu Val 130 135 140		
Arg Ile Thr Ala Ala Thr Arg Val Gly Leu Gly Glu Ser Ser Val Trp 145 150 155 160		
Thr Ser His Arg Thr Pro Lys Ala Thr Ser Val Lys Ala Pro Lys Ser 165 170 175		
Pro Glu Leu His Leu Glu Pro Leu Asn Cys Thr Thr Ile Ser Val Arg 180 185 190		
Trp Gln Gln Asp Val Glu Asp Thr Ala Ala Ile Gln Gly Tyr Lys Leu 195 200 205		
Tyr Tyr Lys Glu Glu Gly Gln Gln Glu Asn Gly Pro Ile Phe Leu Asp 210 215 220		
Thr Lys Asp Leu Leu Tyr Thr Leu Ser Gly Leu Asp Pro Arg Arg Lys 225 230 235 240		
Tyr His Val Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp Gly Tyr Gln 245 250 255		
Ala Asp Gln Thr Val Ser Thr Pro Gly Cys Val Ser Val Arg Asp Arg 260 265 270		
Met Val Pro Pro Pro Pro Pro His His Leu Tyr Ala Lys Ala Asn 275 280 285		
Thr Ser Ser Ser Ile Phe Leu His Trp Arg Arg Pro Ala Phe Thr Ala 290 295 300		
Ala Gln Ile Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val Gly Leu Gln 305 310 315 320		
Asn Ala Ser Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr His Met Leu 325 330 335		
Val Gln Gly Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala Val Arg Leu 340 345 350		
His Val Asp Gln Leu Ser Ser Pro Trp Ser Pro Val Val Tyr His Ser 355 360 365		
Thr Leu Pro Glu Ala Pro Ala Gly Pro Pro Val Gly Val Lys Val Thr 370 375 380		
Leu Ile Glu Asp Asp Thr Ala Leu Val Ser Trp Lys Pro Pro Asp Gly 385 390 395 400		
Pro Glu Thr Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala Ser Arg Lys 405 410 415		
Ala Trp Ile Ala Gly Glu Trp Gln Val Leu His Arg Glu Gly Ala Ile 420 425 430		
Thr Met Ala Leu Leu Glu Asn Leu Val Ala Gly Asn Val Tyr Ile Val 435 440 445		

-continued

Lys Ile Ser Ala Ser Asn Glu Val Gly Glu Gly Pro Phe Ser Asn Ser
 450 455 460
 Val Glu Leu Ala Val Leu Pro Lys Glu Thr Ser Glu Ser Asn Gln Arg
 465 470 475 480
 Pro Lys Arg Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser Gly Tyr Tyr
 485 490 495
 His Leu Asp Gln Lys Ser Met Thr Gly Ile Ala Val Gly Val Gly Ile
 500 505 510
 Ala Leu Thr Cys Ile Leu Ile Cys Val Leu Ile Leu Ile Tyr Arg Ser
 515 520 525
 Lys Ala Arg Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn Gly Thr Gln
 530 535 540
 Gln Leu Pro Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn Glu Val Gly
 545 550 555 560
 Lys Asn Leu Glu Gly Ala Val Gly Asn Glu Glu Ser Leu Met Pro Met
 565 570 575
 Ile Met Pro Asn Ser Phe Ile Asp Ala Lys Val Leu Ser Cys Gly Ile
 580 585 590
 Cys Cys Ile Ser Arg Ser Ser Ile Pro Pro Pro Cys Val Cys Lys Met
 595 600 605
 Tyr Phe Pro Gln Asn Cys Met Leu Asn Val Leu Tyr Gln Tyr Ser Tyr
 610 615 620

<210> SEQ ID NO. 25
 <211> LENGTH: 1644
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 25

atgtcatggt tgaanaatgg aaggaagata cattcgaatg gtagaattaa aatgtacaac 60
 agtaaatggg taattaacca gattattcct gaagatgatg ctatttatca gtgcatggct 120
 gagaatagcc aaggatctat ttatctaga gccagactga ctgtagtgtat gtcagaagac 180
 agaccacgtg ctccctataa tgtacatgct gaaacctagt caagctcagc cattctttta 240
 gcctgggaga ggccacttta taattcagac aaagtcattg cctattctgt acactacatg 300
 aaagcagaag gtttaataaa tgaagagtat caagtagtca tcggaaatga cacaactcat 360
 tatattattg atgacttaga gcctgccagc aattatactt tctacattgt agcatatatg 420
 ccaatgggag ccagccagat gtctgacctat gtgacacaga atactctaga ggatgacccc 480
 agaagaaaa atcatgtgag actcctggct tacaacaaca tagacgatgg ctatcaggca 540
 gatcagactg tcagcactcc aggatgcgtg tctgttcgtg atcgcatggt cctcctccca 600
 ccaccacccc accatctcta tgcgaaggct aacacctcat cttccatctt cotgcactgg 660
 aggaggcctg cattcaccgc tgcacaaatc attaactaca ccatccgctg taatcctggt 720
 ggcctgcaga atgcttcttt ggttctgtac cttcaaacat cagaaactca catgttgggt 780
 caaggtctag aaccaaacac caaatacgaa ttgtccgttc gattacatgt ggcacagctt 840
 tccagtcctt ggagccctgt agtctaccat tctactcttc cagaagcacc agcaggccca 900
 ccagttggag taaaagtgc attaatagag gatgacactg ccctgggttc ttggaaaccc 960
 cctgatggcc cagaaacagt tgtgaccgc tatactatct tatatgcac taggaaggcc 1020
 tggattgcag gagagtggca ggtcttacac cgtgaagggg caataacct ggctttgcta 1080
 gaaaacttgg tagcaggaaa tgtgtacatt gtcaagatat ctgcaccaa tgaggtggga 1140

-continued

```

gaaggaccct tttaaattc tgtggagctg gcagtacttc caaaggaaac ctctgaatca 1200
aatcagaggg ccaagcggtt agattctgct gatgccaag ttatttcagg atattaccat 1260
ctggaccaaa aatcaatgac tggcattgct gtaggtgttg gcatagcctt gacctgcac 1320
ctcatctgtg ttctcatctt gatataccga agtaaagcca ggaaatcatc tgcttccaag 1380
acggcacaga atggaactca acagttacct cgtaccagtg cctccttagc tagtggaat 1440
gaggtaggaa agaacctgga aggagctgta ggaaatgaag aatctttaat gccaatgac 1500
atgccaaca gcttcattga tgcaaagta ctgagctgcy ggatttgctg cataagccgt 1560
tcttcattc ctcctccctg tgtgtgtaaa atgtacttcc cccaaaattg tatgttgaat 1620
gtattatacc aatactctta ttaa 1644

```

<210> SEQ ID NO 26
 <211> LENGTH: 547
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 26

```

Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His Ser Asn Gly Arg Ile
 1             5             10             15
Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln Ile Ile Pro Glu Asp
 20             25             30
Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser Gln Gly Ser Ile Leu
 35             40             45
Ser Arg Ala Arg Leu Thr Val Met Ser Glu Asp Arg Pro Ser Ala
 50             55             60
Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser Ser Ala Ile Leu Leu
 65             70             75             80
Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys Val Ile Ala Tyr Ser
 85             90             95
Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn Glu Glu Tyr Gln Val
100             105             110
Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile Asp Asp Leu Glu Pro
115             120             125
Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr Met Pro Met Gly Ala
130             135             140
Ser Gln Met Ser Asp His Val Thr Gln Asn Thr Leu Glu Asp Asp Pro
145             150             155             160
Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp
165             170             175
Gly Tyr Gln Ala Asp Gln Thr Val Ser Thr Pro Gly Cys Val Ser Val
180             185             190
Arg Asp Arg Met Val Pro Pro Pro Pro Pro His His Leu Tyr Ala
195             200             205
Lys Ala Asn Thr Ser Ser Ser Ile Phe Leu His Trp Arg Arg Pro Ala
210             215             220
Phe Thr Ala Ala Gln Ile Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val
225             230             235             240
Gly Leu Gln Asn Ala Ser Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr
245             250             255
His Met Leu Val Gln Gly Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala
260             265             270
Val Arg Leu His Val Asp Gln Leu Ser Ser Pro Trp Ser Pro Val Val

```

-continued

275	280	285
Tyr His Ser Thr Leu Pro Glu Ala Pro Ala Gly Pro Pro Val Gly Val 290 295 300		
Lys Val Thr Leu Ile Glu Asp Asp Thr Ala Leu Val Ser Trp Lys Pro 305 310 315 320		
Pro Asp Gly Pro Glu Thr Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala 325 330 335		
Ser Arg Lys Ala Trp Ile Ala Gly Glu Trp Gln Val Leu His Arg Glu 340 345 350		
Gly Ala Ile Thr Met Ala Leu Leu Glu Asn Leu Val Ala Gly Asn Val 355 360 365		
Tyr Ile Val Lys Ile Ser Ala Ser Asn Glu Val Gly Glu Gly Pro Phe 370 375 380		
Ser Asn Ser Val Glu Leu Ala Val Leu Pro Lys Glu Thr Ser Glu Ser 385 390 395 400		
Asn Gln Arg Pro Lys Arg Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser 405 410 415		
Gly Tyr Tyr His Leu Asp Gln Lys Ser Met Thr Gly Ile Ala Val Gly 420 425 430		
Val Gly Ile Ala Leu Thr Cys Ile Leu Ile Cys Val Leu Ile Leu Ile 435 440 445		
Tyr Arg Ser Lys Ala Arg Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn 450 455 460		
Gly Thr Gln Gln Leu Pro Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn 465 470 475 480		
Glu Val Gly Lys Asn Leu Glu Gly Ala Val Gly Asn Glu Glu Ser Leu 485 490 495		
Met Pro Met Ile Met Pro Asn Ser Phe Ile Asp Ala Lys Val Leu Ser 500 505 510		
Cys Gly Ile Cys Cys Ile Ser Arg Ser Ser Ile Pro Pro Pro Cys Val 515 520 525		
Cys Lys Met Tyr Phe Pro Gln Asn Cys Met Leu Asn Val Leu Tyr Gln 530 535 540		
Tyr Ser Tyr 545		

<210> SEQ ID NO 27

<211> LENGTH: 2382

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 27

atgtcatggt tgaataatgg aaggaagata cattcgaatg gtagaattaa aatgtacaac	60
agtaaattgg taattaacca gattattcct gaagatgatg ctatttatca gtgcattggt	120
gagaatagcc aaggatctat tttatctaga gccagactga ctgtagtgat gtcagaagac	180
agaccctagt ctccctataa tgtacatgct gaaacctagt caagctcagc cattctttta	240
gcctgggaga ggccacttta taattcagac aaagtcattg cctattctgt acactacatg	300
aaagcagaag gtttaataaa tgaagagtat caagtagtca tcggaaatga cacaactcat	360
tatattattg atgacttaga gcctgccagc aattatactt tctacattgt agcatatatg	420
ccaatgggag ccagccagat gtctgacct gtgacacaga atactctaga ggaattcccc	480
ctgagacctc ctgaaattag ttgacaagt cgaagtccca ctgatattct catctcctgg	540

-continued

ctgccaatcc cagccaaata tcggcggggc caagtgggtgc tgtatcgctt gtctttccgc	600
ctaagtactg agaattcaat ccaagttctg gagctcccg ggaccacgca tgagtacctt	660
ttggaaggcc tgaacctga cagtgtctac ctggttcgga ttactgtgc caccagagt	720
gggctgggag agtcatcagt atggacttca cataggacgc ccaaagctac aagcgtgaaa	780
gcccctaagt ctccagagtt gcatttggag cctctgaact gtaccacctt ttctgtgagg	840
tggcagcaag atgtagagga cacagctgct attcagggtc acaagctgta ctacaaggaa	900
gaagggcagc aggagaatgg gcccatcttc ttggatacca aggacctact ctatactctc	960
agtggcttag accccagaag aaaatatcat gtgagactcc tggcttaca caacatagac	1020
gatggctatc aggagatca gactgtcagc actccaggat gcgtgtctgt tcgtgatcgc	1080
atggtccctc ctccaccacc accccacct ctctatgcga aggctaacac ctcatcttcc	1140
atcttctgc actggaggag gcctgcattc accgctgcac aaatcattaa ctacaccatc	1200
cgctgtaatc ctgttgccct gcagaatgct tctttggttc tgtacctca aacatcagaa	1260
actcacatgt tggttcaagg tctagaacca aacaccaaat acgaatttgc cgttcgatta	1320
catgtggatc agctttccag tccttgagc cctgtagtct accattctac tcttcagaa	1380
gcaccagcag gccaccagc tggagtaaaa gtgacattaa tagaggatga cactgccctg	1440
gtttcttga aacccctga tggccagaa acagttgtga cccgctatac tatcttat	1500
gcacttagga aggcctggat tgcaggagag tggcaggctc tacaccgtga aggggcaata	1560
accatggctt tgctagaaaa cttggtagca ggaatgtgt acattgtcaa gatatctgca	1620
tccaatgagg tgggagaagg acccttttca aattctgtgg agctggcagt acttccaaag	1680
gaaacctctg aatcaaatca gaggcccaag cgtttagatt ctgctgatgc caaagtatt	1740
tcaggatatt accatctgga ccaaaaatca atgactggca ttgctgtagg tgttggcata	1800
gccttgacct gcactctcat ctgtgttctc atcttgatat accgaagtaa agccaggaaa	1860
tcactgtcct ccaagacggc acagaatgga actcaacagt tacctcgta cagtgcctcc	1920
ttagctagtg gaaatgaggt aggaagaac ctggaaggag ctgtaggaaa tgaagaatct	1980
ttaatgcaa tgatcatgcc aaacagcttc attgatgcaa agggaggaac tgacctgata	2040
attaatagct atggctctat aattaaaaac aactctaaga aaaagtgggt ttttttcaa	2100
gactcaaaga agatacaagt tgagcagcct caaagaagat ttactccagc ggtctgcttt	2160
taccagccag gcaccactgt attaatcagt gatgaagact cccctagctc cccaggctcag	2220
acaaccagct tctcaagacc ctttgggtgt gcagctgata cagaacattc agcaaatagt	2280
gaaggcagcc atgagactgg ggattctggg cggttttctc atgagtcгаа cgatgagata	2340
catctgtcct cagttataag taccacaccc cccaacctct ga	2382

<210> SEQ ID NO 28

<211> LENGTH: 793

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 28

Met	Ser	Trp	Leu	Lys	Asn	Gly	Arg	Lys	Ile	His	Ser	Asn	Gly	Arg	Ile
1				5					10					15	
Lys	Met	Tyr	Asn	Ser	Lys	Leu	Val	Ile	Asn	Gln	Ile	Ile	Pro	Glu	Asp
			20					25					30		
Asp	Ala	Ile	Tyr	Gln	Cys	Met	Ala	Glu	Asn	Ser	Gln	Gly	Ser	Ile	Leu
	35						40					45			

-continued

Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu Asp Arg Pro Ser Ala
 50 55 60

Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser Ser Ala Ile Leu Leu
 65 70 75 80

Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys Val Ile Ala Tyr Ser
 85 90 95

Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn Glu Glu Tyr Gln Val
 100 105 110

Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile Asp Asp Leu Glu Pro
 115 120 125

Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr Met Pro Met Gly Ala
 130 135 140

Ser Gln Met Ser Asp His Val Thr Gln Asn Thr Leu Glu Asp Val Pro
 145 150 155 160

Leu Arg Pro Pro Glu Ile Ser Leu Thr Ser Arg Ser Pro Thr Asp Ile
 165 170 175

Leu Ile Ser Trp Leu Pro Ile Pro Ala Lys Tyr Arg Arg Gly Gln Val
 180 185 190

Val Leu Tyr Arg Leu Ser Phe Arg Leu Ser Thr Glu Asn Ser Ile Gln
 195 200 205

Val Leu Glu Leu Pro Gly Thr Thr His Glu Tyr Leu Leu Glu Gly Leu
 210 215 220

Lys Pro Asp Ser Val Tyr Leu Val Arg Ile Thr Ala Ala Thr Arg Val
 225 230 235 240

Gly Leu Gly Glu Ser Ser Val Trp Thr Ser His Arg Thr Pro Lys Ala
 245 250 255

Thr Ser Val Lys Ala Pro Lys Ser Pro Glu Leu His Leu Glu Pro Leu
 260 265 270

Asn Cys Thr Thr Ile Ser Val Arg Trp Gln Gln Asp Val Glu Asp Thr
 275 280 285

Ala Ala Ile Gln Gly Tyr Lys Leu Tyr Tyr Lys Glu Glu Gly Gln Gln
 290 295 300

Glu Asn Gly Pro Ile Phe Leu Asp Thr Lys Asp Leu Leu Tyr Thr Leu
 305 310 315 320

Ser Gly Leu Asp Pro Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr
 325 330 335

Asn Asn Ile Asp Asp Gly Tyr Gln Ala Asp Gln Thr Val Ser Thr Pro
 340 345 350

Gly Cys Val Ser Val Arg Asp Arg Met Val Pro Pro Pro Pro Pro
 355 360 365

His His Leu Tyr Ala Lys Ala Asn Thr Ser Ser Ser Ile Phe Leu His
 370 375 380

Trp Arg Arg Pro Ala Phe Thr Ala Ala Gln Ile Ile Asn Tyr Thr Ile
 385 390 395 400

Arg Cys Asn Pro Val Gly Leu Gln Asn Ala Ser Leu Val Leu Tyr Leu
 405 410 415

Gln Thr Ser Glu Thr His Met Leu Val Gln Gly Leu Glu Pro Asn Thr
 420 425 430

Lys Tyr Glu Phe Ala Val Arg Leu His Val Asp Gln Leu Ser Ser Pro
 435 440 445

Trp Ser Pro Val Val Tyr His Ser Thr Leu Pro Glu Ala Pro Ala Gly
 450 455 460

Pro Pro Val Gly Val Lys Val Thr Leu Ile Glu Asp Asp Thr Ala Leu

-continued

465	470	475	480
Val Ser Trp Lys Pro Pro Asp Gly Pro Glu Thr Val Val Thr Arg Tyr			
485		490	495
Thr Ile Leu Tyr Ala Ser Arg Lys Ala Trp Ile Ala Gly Glu Trp Gln			
500	505		510
Val Leu His Arg Glu Gly Ala Ile Thr Met Ala Leu Leu Glu Asn Leu			
515	520		525
Val Ala Gly Asn Val Tyr Ile Val Lys Ile Ser Ala Ser Asn Glu Val			
530	535		540
Gly Glu Gly Pro Phe Ser Asn Ser Val Glu Leu Ala Val Leu Pro Lys			
545	550	555	560
Glu Thr Ser Glu Ser Asn Gln Arg Pro Lys Arg Leu Asp Ser Ala Asp			
	565	570	575
Ala Lys Val Tyr Ser Gly Tyr Tyr His Leu Asp Gln Lys Ser Met Thr			
	580	585	590
Gly Ile Ala Val Gly Val Gly Ile Ala Leu Thr Cys Ile Leu Ile Cys			
595	600		605
Val Leu Ile Leu Ile Tyr Arg Ser Lys Ala Arg Lys Ser Ser Ala Ser			
610	615		620
Lys Thr Ala Gln Asn Gly Thr Gln Gln Leu Pro Arg Thr Ser Ala Ser			
625	630	635	640
Leu Ala Ser Gly Asn Glu Val Gly Lys Asn Leu Glu Gly Ala Val Gly			
	645	650	655
Asn Glu Glu Ser Leu Met Pro Met Ile Met Pro Asn Ser Phe Ile Asp			
	660	665	670
Ala Lys Gly Gly Thr Asp Leu Ile Ile Asn Ser Tyr Gly Pro Ile Ile			
	675	680	685
Lys Asn Asn Ser Lys Lys Lys Trp Phe Phe Phe Gln Asp Ser Lys Lys			
	690	695	700
Ile Gln Val Glu Gln Pro Gln Arg Arg Phe Thr Pro Ala Val Cys Phe			
705	710	715	720
Tyr Gln Pro Gly Thr Thr Val Leu Ile Ser Asp Glu Asp Ser Pro Ser			
	725	730	735
Ser Pro Gly Gln Thr Thr Ser Phe Ser Arg Pro Phe Gly Val Ala Ala			
	740	745	750
Asp Thr Glu His Ser Ala Asn Ser Glu Gly Ser His Glu Thr Gly Asp			
	755	760	765
Ser Gly Arg Phe Ser His Glu Ser Asn Asp Glu Ile His Leu Ser Ser			
	770	775	780
Val Ile Ser Thr Thr Pro Pro Asn Leu			
785	790		

<210> SEQ ID NO 29

<211> LENGTH: 1887

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 29

atgtcatggt tgaanaatgg aaggaagata cattcgaatg gtagaattaa aatgtacaac	60
agtaaattgg taattaacca gattattcct gaagatgatg ctatttatca gtgcatggct	120
gagaatagcc aaggatctat tttatctaga gccagactga ctgtagtgat gtcagaagac	180
agacccatgt ctccctataa tgtacatgct gaaacatgt caagctcagc cattctttta	240
gcctgggaga ggccacttta taattcagac aaagtcattg cctattctgt acactacatg	300

-continued

```

aaagcagaag gtttaataa tgaagagtat caagtagtca tcggaaatga cacaactcat 360
tatattattg atgacttaga gcctgccagc aattatactt tctacattgt agcatatatg 420
ccaatgggag ccagccagat gtctgacat gtgacacaga atactctaga ggatgacccc 480
agaagaaaat atcatgtgag actcctggct tacaacaaca tagacgatgg ctatcaggca 540
gatcagactg tcagcaactcc aggatgcgtg tctgttcgtg atgcgatggt cctcctccca 600
ccaccacccc accatctcta tgcgaaggct aacacctcat cttccatctt cctgcactgg 660
aggaggcctg cattcaccgc tgcacaaatc attactaca ccatcgctg taatcctgtt 720
ggcctgcaga atgttctttt ggttctgtac cttcaaacat cagaactca catgttggtt 780
caaggctctag aaccaaacac caaatagaa tttgccgttc gattacatgt ggatcagctt 840
tccagtcctt ggagccctgt agtctacat tctactcttc cagaagcacc agcaggccca 900
ccagttggag taaaagtgc attaatagag gatgacactg ccctgggttc ttggaacccc 960
cctgatggcc cagaacacgt tgtgacccgc tatactatct tatatgcac taggaaggcc 1020
tggattgcag gagagtggca ggtcttacac cgtgaagggg caataacat ggctttgcta 1080
gaaaacttgg tagcaggaaa tgtgtacatt gtcaagatat ctgcaccaa tgaggtggga 1140
gaaggaccct tttcaaatc tgtggagctg gcagtacttc caaaggaaac ctctgaatca 1200
aatcagaggc ccaagcgttt agattctgct gatgccaaag tttattcagg atattacat 1260
ctggacaaa aatcaatgac tggcattgct gtagggtgtg gcatagcctt gacctgcac 1320
ctcatctgtg ttctcatctt gatataccga agtaaagcca ggaaatcac tgcttccaag 1380
acggcacaga atggaactca acagttacct cgtaccagt cctccttagc tagtggaaat 1440
gaggtaggaa agaacctgga aggagctgta ggaatgaag aatctttaat gccaatgac 1500
atgccaaaa gcttcattga tgcaaggga ggaactgacc tgataattaa tagctatggt 1560
cctataatta aaacaactc taagaaaaag tggttttttt tccaagactc aaagaagata 1620
caagttgagc agcctcaaag aagatttact ccagcggctt gcttttacca gccaggcacc 1680
actgtattaa tcagtgatga agactccct agctcccccag gtcagacaac cagcttctca 1740
agaccctttg gtgttcagc tgatcacaga cattcagcaa atagtgaagg cagccatgag 1800
actggggatt ctggcggtt ttctcatgag tccaacgat agatacatct gtcctcagtt 1860
ataagtacca cccccccaa cctctga 1887

```

<210> SEQ ID NO 30

<211> LENGTH: 628

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 30

```

Met Ser Trp Leu Lys Asn Gly Arg Lys Ile His Ser Asn Gly Arg Ile
 1           5           10          15
Lys Met Tyr Asn Ser Lys Leu Val Ile Asn Gln Ile Ile Pro Glu Asp
          20          25          30
Asp Ala Ile Tyr Gln Cys Met Ala Glu Asn Ser Gln Gly Ser Ile Leu
          35          40          45
Ser Arg Ala Arg Leu Thr Val Val Met Ser Glu Asp Arg Pro Ser Ala
          50          55          60
Pro Tyr Asn Val His Ala Glu Thr Met Ser Ser Ser Ala Ile Leu Leu
          65          70          75          80
Ala Trp Glu Arg Pro Leu Tyr Asn Ser Asp Lys Val Ile Ala Tyr Ser

```

-continued

85	90	95
Val His Tyr Met Lys Ala Glu Gly Leu Asn Asn Glu Glu Tyr Gln Val 100 105 110		
Val Ile Gly Asn Asp Thr Thr His Tyr Ile Ile Asp Asp Leu Glu Pro 115 120 125		
Ala Ser Asn Tyr Thr Phe Tyr Ile Val Ala Tyr Met Pro Met Gly Ala 130 135 140		
Ser Gln Met Ser Asp His Val Thr Gln Asn Thr Leu Glu Asp Asp Pro 145 150 155 160		
Arg Arg Lys Tyr His Val Arg Leu Leu Ala Tyr Asn Asn Ile Asp Asp 165 170 175		
Gly Tyr Gln Ala Asp Gln Thr Val Ser Thr Pro Gly Cys Val Ser Val 180 185 190		
Arg Asp Arg Met Val Pro Pro Pro Pro Pro His His Leu Tyr Ala 195 200 205		
Lys Ala Asn Thr Ser Ser Ser Ile Phe Leu His Trp Arg Arg Pro Ala 210 215 220		
Phe Thr Ala Ala Gln Ile Ile Asn Tyr Thr Ile Arg Cys Asn Pro Val 225 230 235 240		
Gly Leu Gln Asn Ala Ser Leu Val Leu Tyr Leu Gln Thr Ser Glu Thr 245 250 255		
His Met Leu Val Gln Gly Leu Glu Pro Asn Thr Lys Tyr Glu Phe Ala 260 265 270		
Val Arg Leu His Val Asp Gln Leu Ser Ser Pro Trp Ser Pro Val Val 275 280 285		
Tyr His Ser Thr Leu Pro Glu Ala Pro Ala Gly Pro Pro Val Gly Val 290 295 300		
Lys Val Thr Leu Ile Glu Asp Asp Thr Ala Leu Val Ser Trp Lys Pro 305 310 315 320		
Pro Asp Gly Pro Glu Thr Val Val Thr Arg Tyr Thr Ile Leu Tyr Ala 325 330 335		
Ser Arg Lys Ala Trp Ile Ala Gly Glu Trp Gln Val Leu His Arg Glu 340 345 350		
Gly Ala Ile Thr Met Ala Leu Leu Glu Asn Leu Val Ala Gly Asn Val 355 360 365		
Tyr Ile Val Lys Ile Ser Ala Ser Asn Glu Val Gly Glu Gly Pro Phe 370 375 380		
Ser Asn Ser Val Glu Leu Ala Val Leu Pro Lys Glu Thr Ser Glu Ser 385 390 395 400		
Asn Gln Arg Pro Lys Arg Leu Asp Ser Ala Asp Ala Lys Val Tyr Ser 405 410 415		
Gly Tyr Tyr His Leu Asp Gln Lys Ser Met Thr Gly Ile Ala Val Gly 420 425 430		
Val Gly Ile Ala Leu Thr Cys Ile Leu Ile Cys Val Leu Ile Leu Ile 435 440 445		
Tyr Arg Ser Lys Ala Arg Lys Ser Ser Ala Ser Lys Thr Ala Gln Asn 450 455 460		
Gly Thr Gln Gln Leu Pro Arg Thr Ser Ala Ser Leu Ala Ser Gly Asn 465 470 475 480		
Glu Val Gly Lys Asn Leu Glu Gly Ala Val Gly Asn Glu Glu Ser Leu 485 490 495		
Met Pro Met Ile Met Pro Asn Ser Phe Ile Asp Ala Lys Gly Gly Thr 500 505 510		

-continued

Asp Leu Ile Ile Asn Ser Tyr Gly Pro Ile Ile Lys Asn Asn Ser Lys
 515 520 525
 Lys Lys Trp Phe Phe Phe Gln Asp Ser Lys Lys Ile Gln Val Glu Gln
 530 535 540
 Pro Gln Arg Arg Phe Thr Pro Ala Val Cys Phe Tyr Gln Pro Gly Thr
 545 550 555 560
 Thr Val Leu Ile Ser Asp Glu Asp Ser Pro Ser Ser Pro Gly Gln Thr
 565 570 575
 Thr Ser Phe Ser Arg Pro Phe Gly Val Ala Ala Asp Thr Glu His Ser
 580 585 590
 Ala Asn Ser Glu Gly Ser His Glu Thr Gly Asp Ser Gly Arg Phe Ser
 595 600 605
 His Glu Ser Asn Asp Glu Ile His Leu Ser Ser Val Ile Ser Thr Thr
 610 615 620
 Pro Pro Asn Leu
 625

<210> SEQ ID NO 31
 <211> LENGTH: 3874
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 31

tgcttctcgc gagcgcccg cgcagcacca gctcgcgcgc cgcagagacg ctgcgccgcg	60
cggtgcccga gccggagcgg ggagccaggc tgcgtgcgac cagccgcaga gcagagagcg	120
ccccgggcgg gggccgcaga cggacagggg ctctgggcgg ccggggagca tgcccgcgcg	180
gtacgcgtga atgggcctc ctctgcgacc cctcgcgcgc ctgcgaccgc cggggatgct	240
gtccgcgcgc ctctgctcc tgcgtmtgct cagtctttg ccaggagtgt ggtgctttag	300
cgaactgtct ttgtaaaag aaccacagga tgtaactgtc acaagaaagg acccagtcgt	360
tttagattgc cagggtcacg gagaagttcc tattaaggtc acatggttga aaaatggagc	420
aaaaatgtct gaaaataaac ggatcgaggt tctttctaac ggctctttat acatcagtga	480
ggtggaaggc aggcgaggag agcagtcgga tgaaggattt tatcagtgtc tggcaatgaa	540
caaatatgga gccattctta gtcaaaaagc tcatcttgcc ttatcaacta tttctgcatt	600
tgaagtcag ccaatttcca ctgaggtcca cgaagggtga gttgctcgat ttgcatgcaa	660
gatttcaccc caccctctcg cagtcataac atgggagttc aatcggacaa ctctacctat	720
gactatggac aggataactg ccctaccaac aggagtattg cagatctatg atgtcagcca	780
aagggtattct ggaaattatc gttgtattgc tgccactgta gccaccgcac gtaaaagtat	840
ggaggcctcg ctaactgtga ttccagctaa ggagtcacaa tcttccaca caccarcat	900
tatagcaggt ccacagaaca taacaacatc tcttcacag actgtagttt tggaatgcat	960
ggccacagga aatcccaaac caatcatttc ttggagccgc cttgatcaca aatccattga	1020
tgtctttaat actcgggtac ttggaatgg taatctcatg atatctgatg tcaggctaca	1080
acatgctgga gtatatgttt gtcgggccac taccctggc acacgcaact ttacagttgc	1140
tatggcaact ttaactgtat tagctcctcc ttcatttgtt gaatggccag aaagttaac	1200
aaggcctcga gctggcactg ctgcatttgt gtgtcaggca gaaggaatcc cctctccaa	1260
gatgtcatgg ttgaaaaatg gaaggaagat acattcgaat ggtagaatta aaatgtacaa	1320
cagtaaattg gtaattaacc agattattcc tgaagatgat gctatttata agtgcattgc	1380

-continued

tgagaatagc caaggatcta ttttatctag agccagactg actgtagtga tgtcagaaga	1440
cagaccacagt gctccctata atgtacatgc tgaaccatg tcaagctcag ccattctttt	1500
agcctgggag agggccacttt ataattcaga caaagtcatt gcctattctg tacactacat	1560
gaaagcagaa ggtttaaata atgaagagta tcaagtagtc atcggaatg acacaactca	1620
ttatattatt gatgacttag agcctgccag caattatact ttctacattg tagcatatat	1680
gccaatggga gccagccaga tgtctgacca tgtgacacag aatactctag aggatgttcc	1740
cctgagacct cctgaaatta gtttgacaag tcgaagtccc actgatattc tcattctctg	1800
gctgccaatc ccagccaaat atcggcgggg ccaagtgggt ctgtatcgtg tgtctttccg	1860
cctaagtact gagaattcaa tccaagttct ggagctcccc gggaccacgc atgagtacct	1920
tttggaaggc ctgaaacctg acagtgtcta cctggttcgg attactgctg ccaccagagt	1980
ggggctggga gagtcatcag tatggacttc acataggacg cccaaagcta caagcgtgaa	2040
agcccttaag tctccagagt tgcatttggg gcctctgaac tgtaccacca tttctgtgag	2100
gtggcagcaa gatgtagagg acacagctgc tattcagggc tacaagctgt actacaagga	2160
agaagggcag caggagaatg ggcccatttt ctggataacc aaggacctac tctatactct	2220
cagtggctta gaccccgaaa gaaaatatca tgtgagactc ctggcttaca acaacataga	2280
cgatggctat caggcagatc agactgtcag cactccagga tgcgtgtctg ttcgtgatcg	2340
catggctcct cctccaccac caccaccaca tctctatgag aaggctaaca cctcatcttc	2400
catcttctg cactggagga ggccctgcatt caccgctgca caaatcatta actacaccat	2460
ccgtgtaat cctgttgccc tgcagaatgc ttctttgggt ctgtaccttc aaacatcaga	2520
aactcacatg ttggttcaag gtctagaacc aaacacaaa tacgaatttg ccgttcgatt	2580
acatgtggat cagctttcca gtccttgag ccctgtagtc taccattcta ctctccaga	2640
agcaccagca ggcaccacag ttggagttaa agtgacatta atagaggatg acactgcctt	2700
ggtttcttgg aaacccccctg atggcccaga aacagttgtg acccgctata ctatttata	2760
tgcacttagg aaggcctgga ttgcaggaga gtggcaggtc ttacaccgtg aaggggcaat	2820
aaccatggct ttgctagaaa acctggtagc aggaaatgtg tacattgtca agatatctgc	2880
atccaatgag gtgggagaag gacccttttc aaattctgtg gagctggcag tacttccaaa	2940
ggaaacctct gaatcaaatc agaggcccaa gcgttttagt tctgctgatg ccaaagtta	3000
ttcaggatat taccatctgg accaaaaatc atgactggc attgctgtag gtgttggcat	3060
agccttgacc tgcactctca tctgtgttct catcttgata taccgaagta aagccaggaa	3120
atcatctgct tccaagacgg cacagaatgg aactcaacag ttacctcgta ccagtgcctc	3180
cttagctagt ggaaatgagg taggaaagaa cctggaagga gctgtaggaa atgaagaatc	3240
tttaatgcca atgatcatgc caaacagctt cattgatgca aaggaggaa ctgacctgat	3300
aattaatagc tatggtccta taattaaaaa caactctaag aaaaagtgtt tttttttcca	3360
agactcaaag aagatacaag ttgagcagcc tcaaagaaga tttactccag cggctctgctt	3420
ttaccagcca ggcaccactg tattaatcag tgatgaagac tcccctagct cccaggtca	3480
gacaaccagc tttcaagac ctttgggtgt tgcagctgat acagaacatt cagcaaatag	3540
tgaaggcagc catgagactg gggattctgg gcggttttct catgagtcca acgatgagat	3600
acatctgtcc tcagttataa gtaccacacc ccccaacctc tgattcttcc actggcagtg	3660
attcaggtgg agattccgca ttgaggaagt gtgaagacc tgctgtgtca tctgttagtg	3720
agcagacttc ctcttagtt ctgcagccgc catctgccat gctatgcttt gataaaaatg	3780

-continued

```
atgttccaat ctagacggcc atgtcaggt atttcacca ttaaatctgt tcgaaggaca 3840
atgaacaggg aaccacacac aaaaaaaaaa aaaa 3874
```

What is claimed is:

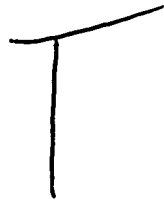
1. An isolated nucleic acid molecule comprising a nucle-¹⁰
otide sequence that:

(b) hybridizes under stringent conditions to the nucleotide
sequence of SEQ ID NO:7 or the complement thereof.

2. An isolated nucleic acid molecule comprising a nucle-
otide sequence encoding the amino acid sequence shown in
SEQ ID NO:8.

(a) encodes the amino acid sequence shown in SEQ ID
NO:8; and

* * * * *

A hand-drawn symbol consisting of a vertical line with a horizontal line intersecting it near the top, and a diagonal line extending from the intersection point towards the upper right.

This Page Blank (uspto)



US006462186B1

(12) **United States Patent**
Donoho et al.

(10) Patent No.: **US 6,462,186 B1**
(45) Date of Patent: **Oct. 8, 2002**

(54) **HUMAN ATPASE PROTEINS AND
POLYNUCLEOTIDES ENCODING THE
SAME**

(75) Inventors: **Gregory Donoho; C. Alexander
Turner, Jr.**, both of The Woodlands;
Erin Hilbun, Spring, all of TX (US);
Michael C. Nehls, Stockdorf (DE);
Glenn Friedrich, Houston, TX (US);
Brian Zambrowicz; Arthur T. Sands,
both of The Woodlands, TX (US)

(73) Assignee: **Lexicon Genetics Incorporated**, The
Woodlands, TX (US)

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/710,092**

(22) Filed: **Nov. 10, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/164,624, filed on Nov. 10,
1999.

(51) Int. Cl.⁷ **C07H 21/04; C12N 9/26**

(52) U.S. Cl. **536/23.2; 435/201**

(58) Field of Search **536/23.2; 435/183,
435/201**

(56) References Cited

U.S. PATENT DOCUMENTS

4,215,051 A	7/1980	Schroeder et al.	435/212
4,376,110 A	3/1983	David et al.	424/1
4,946,778 A	8/1990	Ladner et al.	435/69
5,759,795 A	6/1998	Jubin	435/210
5,837,458 A	11/1998	Minshull et al.	435/6
5,869,336 A	2/1999	Meyer et al.	435/348
5,877,397 A	3/1999	Lonberg et al.	800/200
5,932,444 A	8/1999	Hillman et al.	435/69.1
6,010,852 A	1/2000	Hillman et al.	435/6
6,075,181 A	6/2000	Kucherlapati et al.	800/25

FOREIGN PATENT DOCUMENTS

WO WO 00 58473 A 10/2000

OTHER PUBLICATIONS

Hillier et al. GenBank database—Accession # R60661
(May, 1995).*

Bird et al, 1988, "Single-Chain Antigen-Binding Proteins",
Science 242:423-426.

Bitter et al, 1987, "Expression and Secretion Vectors for
Yeast", Methods in Enzymology 153:516-544.

Colbere-Garapin et al, 1981, "A New Dominant Hybrid
Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol.
150:1-14.

Gautier et al, 1987, "α-DNA IV: α-anomeric and β-anomeric
tetrathymidylates covalently linked to intercalating
oxazopyridocarbazole. Synthesis, physicochemical properties
and poly (rA) binding", Nucleic Acids Research
15(16):6625-6641.

Greenspan et al, 1993, "Idiotypes: structure and immuno-
genicity", FASEB Journal 7:437-444.

Huse et al, 1989 "Generation of a Large Combinatorial
Library of the Immunoglobulin Repertoire in Phage
Lambda", Science 246:1275-1281.

Huston et al, 1988, "Protein engineering of antibody binding
sites: Recovery of specific activity in an anti-digoxin single-
chain Fv analogue produced in *Escherichia coli*", Proc.
Natl. Acad. Sci. USA 85:5879-5883.

Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA
using modified oligonucleotide splints and R Nase H",
FEBS Letters 215(2):327-330.

Inoue et al, 1987, "Synthesis and hybridization studies on
two complementary nona(2'-O-methyl)ribonucleotides",
Nucleic Acids Research 15(15):6131-6149.

Inouye & Inouye, 1985, "Up-promoter mutations in the Ipp
gene of *Escherichia coli*", Nucleic Acids Research
13(9):3101-3110.

Janknecht et al, 1991, "Rapid and efficient purification of
native histidine-tagged protein expressed by recombinant
vaccina virus", PNAS USA 88:8972-8976.

Kohler & Milstein, 1975, "Continuous cultures of fused
cells secreting antibody of predefined specificity", Nature
256:495-497.

Logan et al, 1984, "Adenovirus tripartite leader sequence
enhances translation of mRNAs late after infection", Proc.
Natl. Acad. Sci. USA 81:3655-3659.

Lowy et al, 1980, "Isolation of Transforming DNA: Cloning
the Hamster aprt Gene", Cell 22:817-823.

Morrison et al, 1984, "Chimeric human antibody molecules:
Mouse antigen-binding domains with human constant
region domains", Proc. Natl. Acad. Sci. USA 81:6851-6855.
Mulligan & Berg, 1981, "Selection for animal cells that
express the *Escherichia coli* gene coding for xanthine-gua-
nine phosphoribosyltransferase", Proc. Natl. Acad. Sci. USA
78(4):2072-2076.

Neuberger et al, 1984, "Recombinant antibodies possessing
novel effector functions", Nature 312:604-608.

Nisonoff, 1991, "Idiotypes: Concepts and Applications", J.
of Immunology 147:2429-2438.

O'Hare et al, 1981, "Transformation of mouse fibroblasts to
methotrexate resistance by a recombinant plasmid express-
ing a prokaryotic dihydrofolate reductase", Proc. Natl. Acad.
Sci. USA 78(3):1527-1531.

Ruther et al, 1983, "Easy identification of cDNA clones",
EMBO Journal 2(10):1791-1794.

Santerre et al, 1984, "Expression of prokaryotic genes for
hygromycin B and G418 resistance as dominant-selection
markers in mouse L cells", Gene 30:147-156.

Sarin et al, 1988, "Inhibition of acquired immunodeficiency
syndrome virus by oligodeoxynucleoside methylphospho-
nates", Proc. Natl. Acad. Sci. USA 85:7448-7451.

(List continued on next page.)

Primary Examiner—Tekchand Saidha

Assistant Examiner—Yong Pak

(57) ABSTRACT

Novel human polynucleotide and polypeptide sequences are
disclosed that can be used in therapeutic, diagnostic, and
pharmacogenomic applications.

3 Claims, No Drawings

OTHER PUBLICATIONS

- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", J. Virol. 46(2):584-593.
- Stein et al, 1988, "Physiochemical properties of phosphorothioate oligodeoxynucleotides", Nucleic Acids Research 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", Proc. Natl. Acad. Sci. USA 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", Nature 314:452-454.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", J. Biol. Chemistry 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature 341:544-546.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", Proc. Natl. Acad. Sci. USA 77(6):3567-3570.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cell", Cell 11:223-232.
- EMBL Database, Heidelberg, FRG accession No. AW024552, Sep. 14, 1999, NCI-CGAP: "wu77c05.x1 NCI_CGAP_Kid 3 Homo sapiens cDNA clone IMAGE: 252056 3' similar to WP: W09D10.2 CE16563 ATPASE, mRNA sequence" XP002165685.
- Halleck, M.S. et al., 1998, "Multiple Members of a Third Subfamily of P-Type ATPases Identified by Genomic Sequences and ESTs", Genome Research 8(4): 354-361. XP002132690, Accession No.:AF011337.
- Nagase, T. et al, 2000, "Prediction of the Coding Sequences of Unidentified Human Genes. XVII. The Complete Sequences of 100 New cDNA Clones from Brain Which Code for Large Proteins in vitro", DNA Research 7(2):143-150. XP000983090, clone XP000983090.

* cited by examiner

1

HUMAN ATPASE PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application No. 60/164,624 which was filed on Nov. 10, 1999 and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins that share sequence similarity with animal ATPase proteins. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed genes, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed genes that can be used for diagnosis, drug screening, clinical trial monitoring and the treatment of diseases and disorders.

2. BACKGROUND OF THE INVENTION

ATPases are proteins that mediate, facilitate, or "power" a wide variety of chemical processes within the cell. For example, ATPases have been associated with enzymatic, catabolic, and metabolic processes as well as transport mechanisms, blood coagulation, phagocytosis, etc.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins, and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPS) described for the first time herein share structural similarity with animal ATPases.

The novel human nucleic acid sequences described herein, encode alternative proteins/open reading frames (ORFs) of 972, 124, 1,056, 208, 1,270, 422, 1,426, and 578 amino acids in length (see SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16 respectively).

The invention also encompasses agonists and antagonists of the described NHPS, including small molecules, large molecules, mutant NHPs, or portions thereof that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHP genes (e.g., expression constructs that place the described gene under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" (which can be conditional) that do not express a functional NHP. A knockout ES cell line has been produced that contains a gene trap mutation in the murine ortholog of the described locus.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

2

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequences of the described NHP ORFs that encode the described NHP amino acid sequences. SEQ ID NO:17 describes a NHP ORF as well as flanking 5' and 3' sequences.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPS, described for the first time herein, are novel proteins that are expressed in, inter alia, human cell lines, predominantly in human kidney and placenta, as well as human fetal brain, brain, pituitary, cerebellum, spinal cord, thymus, spleen, lymph node, bone marrow, trachea, fetal liver, prostate, testis, thyroid, adrenal gland, salivary gland, stomach, small intestine, colon, uterus, mammary gland, adipose, esophagus, bladder, cervix, rectum, ovary, fetal kidney, fetal lung and gene trapped human cells.

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of the NHPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of an NHP, or one of its domains (e.g., a receptor or ligand binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of a DNA sequence that encodes and expresses an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, supra), yet still encodes a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling,

directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP gene nucleotide sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the NHP sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described NHP polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 18, and preferably about 25, nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences may begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine,

5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the

intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene. The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, connective tissue disorders, infertility, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue can be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869, 336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the human cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of the NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies,

antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor for an NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding the NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP receptor. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 The NHP Sequences

The cDNA sequences and the corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing. The NHP nucleotides were obtained from clustered human gene trapped sequences, ESTs and a human placenta cDNA library (Edge Biosystems, Gaithersburg, Md.). The described sequences share structural similarity with calcium transporting ATPases and aminophospholipid transporters.

5.2 NHPS and NHP Polypeptides

NHPs, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include, but are not limited to, the generation of antibodies, as reagents in diagnostic assays, for the identification of other cellular gene products related to a NHP, as reagents in assays for screening for compounds that can be as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease. Given the similarity information and expression data, the described NHPs can be targeted (by drugs, oligos, antibodies, etc.) in order to treat disease, or to therapeutically augment the efficacy of therapeutic agents.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP genes. The NHPs typically display initiator methionines in DNA sequence contexts consistent with a translation initiation site.

The NHP amino acid sequences of the invention include the amino acid sequence presented in the Sequence Listing

as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a substrate of a NHP, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, transport, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where, as in the present instance, the NHP peptide or polypeptide is thought to be membrane protein, the hydrophobic regions of the protein can be excised and the resulting soluble peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, *in situ*. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP

nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Pharmacia or American Type Culture Collection) can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polydrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own

initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygromycin, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} -nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

5.3 Antibodies to NHP Products

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab)'_2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with the NHP, an NHP peptide (e.g., one corresponding to the functional domain of an NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor

et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mabs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Pat. Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab)'_2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab)'_2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 17

<210> SEQ ID NO 1

<211> LENGTH: 2919

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atgactgagg ctctccaatg ggccagatat cactggcgac ggctgatcag aggtgcaacc      60
agggatgatg attcaggggc atacaactat tcctcgttgc tcgcctgttg gcgcaagtcc      120
tctcagatcc cttaactgtc aggaaggcac cggattgttg tccccacat ccagcccttc      180
aaggatgagt atgagaagtt ctccggagcc tatgtgaaca atcgaatacg aacaacaaag      240
tacacacttc tgaattttgt gccaaagaaat ttatttgaac aatttcacag agctgccaat      300
ttatatttcc tgttcctagt tgtcctgaac tgggtacctt tggtagaagc cttccaaaag      360
gaaatcacca tgttgccctc ggtgggtggc cttacaatta tcgcaattaa agatggcctg      420
gaagattatc ggaaatacaa aattgacaaa cagatcaata atttaataac taaagtttat      480
agtaggaaag agaaaaaata cattgaccga tgctggaaag acgttactgt tggggacttt      540
attcgctctc cctgcaacga ggtcatccct gcagacatgg tactactctt ttccactgat      600
ccagatggaa tctgtccatc tgagacttct ggtcttgatg gagagagcaa tttaaaacag      660
aggcaggttg ttccggggata tgcagaacag gactctgaag ttgatcctga gaagttttcc      720
agtaggatag aatgtgaaag cccaaacaat gacctcagca gattccgagg ctctctagaa      780
cattccaaca aagaacgcgt gggctctcagt aaagaaaatt tgttgcttag aggatgcacc      840
attagaaaca cagaggctgt tgtgggcatt gtggtttatg caggccatga aaccaagca      900
atgctgaaca acagtgggcc acggtataag cgacagcaat tagaaagaag agcaaacaca      960
gatgtcctct ggtgtgcatc gcttctggtc ataattgtgt taactggcgc agtaggtcat      1020
ggaatctggc tgagcaggta tgaaaagatg cttttttca atgttccga gcctgatgga      1080
catatcatat caccactgtt ggcaggattt tatatgtttt ggaccatgat ctttttgta      1140
caggctcttg tctctatttc tctctatggt tccatcgaaa ttgtgaagct tggacaataa      1200
tatttcattc aaagtgatgt ggatttctac aatgaaaaaa tggattctat tgttcagtgc      1260
cgagccctga acatcgccga ggatctggga cagattcagt acctcttttc cgataagaca      1320
ggaaccctca ctgagaataa gatggttttt cgaagatgta gtgtggcagg atttgattac      1380
tgccatgaag aaaatgccag gaggttgag tcctatcagg aagctgtctc tgaagatgaa      1440
gattttatag acacagtcag tggttccctc agcaatatgg caaaaccgag agcccccagc      1500
tgcaggacag ttcataatgg gcctttggga aataagccct caaatcatct tgcctgggagc      1560
tcttttactc taggaagtgg agaaggagcc agtgaagtgc ctcatccag acaggctgct      1620
ttcagtagcc ccattgaaac agacgtggta ccagacacca ggcttttaga caaathtagt      1680
cagattacac ctcggtctct tatgccacta gatgagacca tccaaaaatc accaatggaa      1740
actttgtaca ttatcgactt tticattgca ttggcaattt gcaacacagt agtggtttct      1800
gtccttaacc aaccccgaca aaagatcaga cacccttcac tggggggggt gcccataaag      1860
tctttggaag agattaaaag tcttttccag agatggtctg tccgaagatc aagttctcca      1920
tcgcttaaca gtgggaaaga gccatcttct ggagttccaa acgcctttgt ggcagactc      1980
cctctcttta gtcgaaatgaa accagcttca cctgtggagg aagaggtctc ccagggtgtg      2040

```

-continued

```

gagagccccc agtgcctccag tagctcagct tgctgcacag aaacagagaa acaacacggt 2100
gatgcaggcc tctgaatgg caaggcagag tccctccctg gacagccatt ggcctgcaac 2160
ctgtgttatg aggcagagag cccagacgaa ggggccttag tgtatgccgc cagggttac 2220
caatgcactt tacggctctg gacaccagag caggctcatg tggactttgc tgctttggga 2280
ccattaacat ttcaactcct acacatcctg ccttttgact cagtaagaaa aagaatgtct 2340
gttggtgtcc gacaccctct ttccaatcaa gttgtggtgt atacgaaagg cgctgattct 2400
gtgatcatgg agttactgtc ggtggcttcc ccagatggag caagtctgga gaaacaacag 2460
atgatagtaa gggagaaaac ccagaagcac ttggatgact atgccaaaca aggccttcgt 2520
actttatgta tagcaaagaa ggtcatgagt gacactgaat atgcagagtg gctgaggaat 2580
cattttttag ctgaaaccag cattgacaac agggaagaat tactacttga atctgccatg 2640
aggttggaag acaaacttac attacttggt gctactggca ttgaagaccg tctgcaggag 2700
ggagtcctct aatctataga agctcttcac aaagcgggca tcaagatctg gatgctgaca 2760
ggggacaagc agggagacagc tgtcaacata gcttatgcat gcaactact ggagccagat 2820
gacaagcttt ttatcctcaa taccctaaagt aaagtgcgta tattgagatt aaatctgttc 2880
ttctgtattt tcaaaggcat tggaacattt gagatttga 2919

```

```

<210> SEQ ID NO 2
<211> LENGTH: 972
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 2

```

```

Met Thr Glu Ala Leu Gln Trp Ala Arg Tyr His Trp Arg Arg Leu Ile
 1          5          10          15
Arg Gly Ala Thr Arg Asp Asp Asp Ser Gly Pro Tyr Asn Tyr Ser Ser
 20          25          30
Leu Leu Ala Cys Gly Arg Lys Ser Ser Gln Ile Pro Lys Leu Ser Gly
 35          40          45
Arg His Arg Ile Val Val Pro His Ile Gln Pro Phe Lys Asp Glu Tyr
 50          55          60
Glu Lys Phe Ser Gly Ala Tyr Val Asn Asn Arg Ile Arg Thr Thr Lys
 65          70          75          80
Tyr Thr Leu Leu Asn Phe Val Pro Arg Asn Leu Phe Glu Gln Phe His
 85          90          95
Arg Ala Ala Asn Leu Tyr Phe Leu Phe Leu Val Val Leu Asn Trp Val
100          105          110
Pro Leu Val Glu Ala Phe Gln Lys Glu Ile Thr Met Leu Pro Leu Val
115          120          125
Val Val Leu Thr Ile Ile Ala Ile Lys Asp Gly Leu Glu Asp Tyr Arg
130          135          140
Lys Tyr Lys Ile Asp Lys Gln Ile Asn Asn Leu Ile Thr Lys Val Tyr
145          150          155          160
Ser Arg Lys Glu Lys Lys Tyr Ile Asp Arg Cys Trp Lys Asp Val Thr
165          170          175
Val Gly Asp Phe Ile Arg Leu Ser Cys Asn Glu Val Ile Pro Ala Asp
180          185          190
Met Val Leu Leu Phe Ser Thr Asp Pro Asp Gly Ile Cys His Ile Glu
195          200          205
Thr Ser Gly Leu Asp Gly Glu Ser Asn Leu Lys Gln Arg Gln Val Val

```

-continued

210	215	220
Arg Gly Tyr Ala Glu	Gln Asp Ser Glu Val	Asp Pro Glu Lys Phe Ser
225	230	235 240
Ser Arg Ile Glu Cys	Glu Ser Pro Asn Asn Asp	Leu Ser Arg Phe Arg
245	250	255
Gly Phe Leu Glu His	Ser Asn Lys Glu Arg Val	Gly Leu Ser Lys Glu
260	265	270
Asn Leu Leu Leu Arg	Gly Cys Thr Ile Arg Asn	Thr Glu Ala Val Val
275	280	285
Gly Ile Val Val Tyr	Ala Gly His Glu Thr Lys	Ala Met Leu Asn Asn
290	295	300
Ser Gly Pro Arg Tyr	Lys Arg Ser Lys Leu Glu	Arg Arg Ala Asn Thr
305	310	315 320
Asp Val Leu Trp Cys	Val Met Leu Leu Val	Ile Met Cys Leu Thr Gly
325	330	335
Ala Val Gly His Gly	Ile Trp Leu Ser Arg Tyr	Glu Lys Met His Phe
340	345	350
Phe Asn Val Pro Glu	Pro Asp Gly His Ile Ile	Ser Pro Leu Leu Ala
355	360	365
Gly Phe Tyr Met Phe	Trp Thr Met Ile Ile Leu	Leu Gln Val Leu Ile
370	375	380
Pro Ile Ser Leu Tyr	Val Ser Ile Glu Ile Val	Lys Leu Gly Gln Ile
385	390	395 400
Tyr Phe Ile Gln Ser	Asp Val Asp Phe Tyr Asn	Glu Lys Met Asp Ser
405	410	415
Ile Val Gln Cys Arg	Ala Leu Asn Ile Ala Glu	Asp Leu Gly Gln Ile
420	425	430
Gln Tyr Leu Phe Ser	Asp Lys Thr Gly Thr Leu	Thr Glu Asn Lys Met
435	440	445
Val Phe Arg Arg Cys	Ser Val Ala Gly Phe Asp	Tyr Cys His Glu Glu
450	455	460
Asn Ala Arg Arg Leu	Glu Ser Tyr Gln Glu Ala	Val Ser Glu Asp Glu
465	470	475 480
Asp Phe Ile Asp Thr	Val Ser Gly Ser Leu Ser	Asn Met Ala Lys Pro
485	490	495
Arg Ala Pro Ser Cys	Arg Thr Val His Asn Gly	Pro Leu Gly Asn Lys
500	505	510
Pro Ser Asn His Leu	Ala Gly Ser Ser Phe Thr	Leu Gly Ser Gly Glu
515	520	525
Gly Ala Ser Glu Val	Pro His Ser Arg Gln Ala	Ala Phe Ser Ser Pro
530	535	540
Ile Glu Thr Asp Val	Val Pro Asp Thr Arg Leu	Leu Asp Lys Phe Ser
545	550	555 560
Gln Ile Thr Pro Arg	Leu Phe Met Pro Leu Asp	Glu Thr Ile Gln Asn
565	570	575
Pro Pro Met Glu Thr	Leu Tyr Ile Ile Asp Phe	Phe Ile Ala Leu Ala
580	585	590
Ile Cys Asn Thr Val	Val Val Ser Ala Pro Asn	Gln Pro Arg Gln Lys
595	600	605
Ile Arg His Pro Ser	Leu Gly Leu Pro Ile Lys	Ser Leu Glu Glu
610	615	620
Ile Lys Ser Leu Phe	Gln Arg Trp Ser Val Arg	Arg Ser Ser Ser Pro
625	630	635 640

-continued

Ser Leu Asn Ser Gly Lys Glu Pro Ser Ser Gly Val Pro Asn Ala Phe
 645 650 655
 Val Ser Arg Leu Pro Leu Phe Ser Arg Met Lys Pro Ala Ser Pro Val
 660 665 670
 Glu Glu Glu Val Ser Gln Val Cys Glu Ser Pro Gln Cys Ser Ser Ser
 675 680 685
 Ser Ala Cys Cys Thr Glu Thr Glu Lys Gln His Gly Asp Ala Gly Leu
 690 695 700
 Leu Asn Gly Lys Ala Glu Ser Leu Pro Gly Gln Pro Leu Ala Cys Asn
 705 710 715 720
 Leu Cys Tyr Glu Ala Glu Ser Pro Asp Glu Ala Ala Leu Val Tyr Ala
 725 730 735
 Ala Arg Ala Tyr Gln Cys Thr Leu Arg Ser Arg Thr Pro Glu Gln Val
 740 745 750
 Met Val Asp Phe Ala Ala Leu Gly Pro Leu Thr Phe Gln Leu Leu His
 755 760 765
 Ile Leu Pro Phe Asp Ser Val Arg Lys Arg Met Ser Val Val Val Arg
 770 775 780
 His Pro Leu Ser Asn Gln Val Val Val Tyr Thr Lys Gly Ala Asp Ser
 785 790 795 800
 Val Ile Met Glu Leu Leu Ser Val Ala Ser Pro Asp Gly Ala Ser Leu
 805 810 815
 Glu Lys Gln Gln Met Ile Val Arg Glu Lys Thr Gln Lys His Leu Asp
 820 825 830
 Asp Tyr Ala Lys Gln Gly Leu Arg Thr Leu Cys Ile Ala Lys Lys Val
 835 840 845
 Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 850 855 860
 Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
 865 870 875 880
 Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 885 890 895
 Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 900 905 910
 Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 915 920 925
 Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 930 935 940
 Ile Leu Asn Thr Gln Ser Lys Val Arg Ile Leu Arg Leu Asn Leu Phe
 945 950 955 960
 Phe Cys Ile Phe Lys Gly Ile Gly Thr Phe Glu Ile
 965 970

<210> SEQ ID NO 3

<211> LENGTH: 375

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 3

atgagtgaca ctgaatatgc agagtggctg aggaatcatt ttttagctga aaccagcatt 60
 gacaacaggg aagaattact acttgaatct gccatgaggt tggagaacaa acttacatta 120
 cttggtgcta ctggcattga agaccgtctg caggaggag tccctgaatc tatagaagct 180
 cttcacaag cgggcatcaa gatctggatg ctgacagggg acaagcagga gacagctgtc 240

-continued

aacatagctt atgcatgcaa actactggag ccagatgaca agctttttat cctcaatacc 300
 caaagtaaag tgcgtatatt gagattaaat ctgttcttct gtattttcaa aggcatgga 360
 acatttgaga tttga 375

<210> SEQ ID NO 4
 <211> LENGTH: 124
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 4

Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 1 5 10 15
 Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
 20 25 30
 Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 35 40 45
 Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 50 55 60
 Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 65 70 75 80
 Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 85 90 95
 Ile Leu Asn Thr Gln Ser Lys Val Arg Ile Leu Arg Leu Asn Leu Phe
 100 105 110
 Phe Cys Ile Phe Lys Gly Ile Gly Thr Phe Glu Ile
 115 120

<210> SEQ ID NO 5
 <211> LENGTH: 3171
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 5

atgactgagg ctctccaatg ggccagatat caotggcgac ggctgatcag aggtgcaacc 60
 agggatgatg attcagggcc atacaactat tctcgttgc tcgcctgtgg gcgcaagtcc 120
 tctcagatcc ctactactgtc aggaaggcac cggattgttg ttccccacat ccagcccttc 180
 aaggatgagt atgagaagtt ctccggagcc tatgtgaaca atcgaatacg aacaacaaag 240
 tacacacttc tgaattttgt gccaagaaat ttatttgaac aatttcacag agctgccaat 300
 ttatatttcc tgttcctagt tgtcctgaac tgggtacctt tggtagaagc cttccaaaag 360
 gaaatcacca tgttgctctc ggtgggggtc cttacaatta tcgcaattaa agatggcctg 420
 gaagattatc ggaaatacaa aattgacaaa cagatcaata atttaataac taaagtttat 480
 agtaggaaa agaaaaaata cattgaccga tgcaggaaag acgttactgt tggggacttt 540
 attcgctct cctgcaacga ggtcatccct gcagacatgg tactactctt ttccactgat 600
 ccagatggaa tctgtcacat tgagacttct ggtcttgatg gagagagcaa tttaaaacag 660
 aggcagggtg ttcggggata tgcagaacag gactctgaag ttgatcctga gaagttttcc 720
 agtaggatag aatgtgaaag cccaaacaat gacctcagca gattccgagg cttcctagaa 780
 cattccaaca aagaacgcgt gggctctcagt aaagaaaatt tgttgcttag aggatgcacc 840
 attagaaca cagaggctgt tgtgggcatt gtggtttatg caggccatga aaccaaagca 900
 atgctgaaca acagtgggcc acgttataag cgcagcaaat tagaagaag agcaaacaca 960

-continued

gatgtcctct ggtgtgtcat gcttctggtc ataatgtgct taactggcgc agtaggtcat	1020
ggaatctggc tgagcaggta tgaaaagatg ctttttttca atgttcccgga gcctgatgga	1080
catatcatat caccactgtt ggcaggattt tatatgtttt ggaccatgat ctttttgta	1140
caggtcttga ttcttatctt tctctatggt tccatcgaaa ttgtgaagct tggacaaata	1200
tatttcattc aaagtgatgt ggatttctac aatgaaaaaa tggattctat tgttcagtgc	1260
cgagccctga acatcgccga ggatctggga cagattcagt acctcttttc cgataagaca	1320
ggaaccctca ctgagaataa gatggttttt cgaagatgta gtgtggcagg atttgattac	1380
tgccatgaag aaaatgccag gaggttgag tcctatcagg aagctgtctc tgaagatgaa	1440
gattttatag acacagtcag tggttccctc agcaatatgg caaaaccgag agcccccagc	1500
tgcaggacag ttcataatgg gcctttggga aataagccct caaatcatct tgctgggagc	1560
tcttttactc taggaagtgg agaaggagcc agtgaagtgc ctcatccag acaggctgct	1620
ttcagtagcc ccattgaaac agacgtggta ccagacacca ggcttttaga caaattagt	1680
cagattacac ctcggtctct tatgccacta gatgagacca tccaaaatcc accaatggaa	1740
actttgtaca ttatcgactt ttctattgca ttggcaattt gcaacacagt agtggtttct	1800
gtcctaacc aaccccgaca aaagatcaga cacccttcac tgggggggtt gccattaag	1860
tctttggaag agattaaaag tcttttccag agatggctg tccgaagatc aagttctcca	1920
tcgcttaaca gtgggaaaga gccatcttct ggagttccaa acgcctttgt gacgagactc	1980
cctctcttta gtcgaaatgaa accagcttca cctgtggagg aagaggtctc ccagggtgtg	2040
gagagccccc agtgcctcag tagctcagct tgctgcacag aaacagagaa acaacacggt	2100
gatgcaggcc tcctgaatgg caaggcagag tccctccctg gacagccatt ggcctgcaac	2160
ctgtgttatg aggcgagag cccagacgaa gcggccttag tgatgccgc cagggtctac	2220
caatgcactt tacggctcgc gacaccagag caggctcatg tggactttgc tgccttgga	2280
ccattaacat ttcaactcct acacatcctg cccttgact cagtaagaaa aagaatgtct	2340
gttgtgtgct gacaccctct ttccaatcaa gttgtggtg atacgaaagg cgctgattct	2400
gtgatcatgg agttactgtc ggtggcttcc ccagatggag caagtctgga gaaacaacag	2460
atgatagtaa ggggaaaac ccagaagcac ttggatgact atgccaaaca aggccttcgt	2520
actttatgta tagcaaaaga ggtcatgagt gacactgaat atgcagagt gctgaggaat	2580
catttttttag ctgaaaccag cattgacaac agggaagaat tactacttga atctgccatg	2640
aggttgagga acaaaacttac attacttggg gctactggca ttgaagaccg tctgcaggag	2700
ggagtccttg aatctataga agctcttcac aaagcgggca tcaagatctg gatgctgaca	2760
ggggacaagc aggagacagc tgtcaacata gcttatgcat gcaaaactact ggagccagat	2820
gacaagcttt ttatcctcaa taccctaaagt aaagatgcct gtgggatgct gatgagcaca	2880
attttgaaag aacttcagaa gaaaactcaa gccctgccag agcaagtgtc attaatgaa	2940
gatttacttc agcctcctgt ccccgaggac tcagggttac gagctggact cattatcact	3000
gggaagaccc tggagtgtgc cctgcaagaa agtctgcaaa agcagttcct ggaactgaca	3060
tcttggtgtc aagctgtggt ctgctgcga gccacaccgc tgcagaaaag tgaagtgtg	3120
aaattgggtc gcagccatct ccaggatg acccttgcta ttggtgagtg a	3171

<210> SEQ ID NO 6

<211> LENGTH: 1056

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 6

```

Met Thr Glu Ala Leu Gln Trp Ala Arg Tyr His Trp Arg Arg Leu Ile
 1           5           10           15
Arg Gly Ala Thr Arg Asp Asp Asp Ser Gly Pro Tyr Asn Tyr Ser Ser
          20           25           30
Leu Leu Ala Cys Gly Arg Lys Ser Ser Gln Ile Pro Lys Leu Ser Gly
          35           40           45
Arg His Arg Ile Val Val Pro His Ile Gln Pro Phe Lys Asp Glu Tyr
          50           55           60
Glu Lys Phe Ser Gly Ala Tyr Val Asn Asn Arg Ile Arg Thr Thr Lys
          65           70           75           80
Tyr Thr Leu Leu Asn Phe Val Pro Arg Asn Leu Phe Glu Gln Phe His
          85           90           95
Arg Ala Ala Asn Leu Tyr Phe Leu Phe Leu Val Val Leu Asn Trp Val
          100          105          110
Pro Leu Val Glu Ala Phe Gln Lys Glu Ile Thr Met Leu Pro Leu Val
          115          120          125
Val Val Leu Thr Ile Ile Ala Ile Lys Asp Gly Leu Glu Asp Tyr Arg
          130          135          140
Lys Tyr Lys Ile Asp Lys Gln Ile Asn Asn Leu Ile Thr Lys Val Tyr
          145          150          155          160
Ser Arg Lys Glu Lys Lys Tyr Ile Asp Arg Cys Trp Lys Asp Val Thr
          165          170          175
Val Gly Asp Phe Ile Arg Leu Ser Cys Asn Glu Val Ile Pro Ala Asp
          180          185          190
Met Val Leu Leu Phe Ser Thr Asp Pro Asp Gly Ile Cys His Ile Glu
          195          200          205
Thr Ser Gly Leu Asp Gly Glu Ser Asn Leu Lys Gln Arg Gln Val Val
          210          215          220
Arg Gly Tyr Ala Glu Gln Asp Ser Glu Val Asp Pro Glu Lys Phe Ser
          225          230          235          240
Ser Arg Ile Glu Cys Glu Ser Pro Asn Asn Asp Leu Ser Arg Phe Arg
          245          250          255
Gly Phe Leu Glu His Ser Asn Lys Glu Arg Val Gly Leu Ser Lys Glu
          260          265          270
Asn Leu Leu Leu Arg Gly Cys Thr Ile Arg Asn Thr Glu Ala Val Val
          275          280          285
Gly Ile Val Val Tyr Ala Gly His Glu Thr Lys Ala Met Leu Asn Asn
          290          295          300
Ser Gly Pro Arg Tyr Lys Arg Ser Lys Leu Glu Arg Arg Ala Asn Thr
          305          310          315          320
Asp Val Leu Trp Cys Val Met Leu Leu Val Ile Met Cys Leu Thr Gly
          325          330          335
Ala Val Gly His Gly Ile Trp Leu Ser Arg Tyr Glu Lys Met His Phe
          340          345          350
Phe Asn Val Pro Glu Pro Asp Gly His Ile Ile Ser Pro Leu Leu Ala
          355          360          365
Gly Phe Tyr Met Phe Trp Thr Met Ile Ile Leu Leu Gln Val Leu Ile
          370          375          380
Pro Ile Ser Leu Tyr Val Ser Ile Glu Ile Val Lys Leu Gly Gln Ile
          385          390          395          400
Tyr Phe Ile Gln Ser Asp Val Asp Phe Tyr Asn Glu Lys Met Asp Ser

```

-continued

405	410	415
Ile Val Gln Cys Arg Ala Leu Asn	Ile Ala Glu Asp Leu Gly Gln Ile	
420	425	430
Gln Tyr Leu Phe Ser Asp Lys Thr	Gly Thr Leu Thr Glu Asn Lys Met	
435	440	445
Val Phe Arg Arg Cys Ser Val Ala Gly Phe Asp Tyr Cys His Glu Glu		
450	455	460
Asn Ala Arg Arg Leu Glu Ser Tyr Gln Glu Ala Val Ser Glu Asp Glu		
465	470	475
Asp Phe Ile Asp Thr Val Ser Gly Ser Leu Ser Asn Met Ala Lys Pro		
485	490	495
Arg Ala Pro Ser Cys Arg Thr Val His Asn Gly Pro Leu Gly Asn Lys		
500	505	510
Pro Ser Asn His Leu Ala Gly Ser Ser Phe Thr Leu Gly Ser Gly Glu		
515	520	525
Gly Ala Ser Glu Val Pro His Ser Arg Gln Ala Ala Phe Ser Ser Pro		
530	535	540
Ile Glu Thr Asp Val Val Pro Asp Thr Arg Leu Leu Asp Lys Phe Ser		
545	550	555
Gln Ile Thr Pro Arg Leu Phe Met Pro Leu Asp Glu Thr Ile Gln Asn		
565	570	575
Pro Pro Met Glu Thr Leu Tyr Ile Ile Asp Phe Phe Ile Ala Leu Ala		
580	585	590
Ile Cys Asn Thr Val Val Val Ser Ala Pro Asn Gln Pro Arg Gln Lys		
595	600	605
Ile Arg His Pro Ser Leu Gly Gly Leu Pro Ile Lys Ser Leu Glu Glu		
610	615	620
Ile Lys Ser Leu Phe Gln Arg Trp Ser Val Arg Arg Ser Ser Ser Pro		
625	630	635
Ser Leu Asn Ser Gly Lys Glu Pro Ser Ser Gly Val Pro Asn Ala Phe		
645	650	655
Val Ser Arg Leu Pro Leu Phe Ser Arg Met Lys Pro Ala Ser Pro Val		
660	665	670
Glu Glu Glu Val Ser Gln Val Cys Glu Ser Pro Gln Cys Ser Ser Ser		
675	680	685
Ser Ala Cys Cys Thr Glu Thr Glu Lys Gln His Gly Asp Ala Gly Leu		
690	695	700
Leu Asn Gly Lys Ala Glu Ser Leu Pro Gly Gln Pro Leu Ala Cys Asn		
705	710	715
Leu Cys Tyr Glu Ala Glu Ser Pro Asp Glu Ala Ala Leu Val Tyr Ala		
725	730	735
Ala Arg Ala Tyr Gln Cys Thr Leu Arg Ser Arg Thr Pro Glu Gln Val		
740	745	750
Met Val Asp Phe Ala Ala Leu Gly Pro Leu Thr Phe Gln Leu Leu His		
755	760	765
Ile Leu Pro Phe Asp Ser Val Arg Lys Arg Met Ser Val Val Val Arg		
770	775	780
His Pro Leu Ser Asn Gln Val Val Val Tyr Thr Lys Gly Ala Asp Ser		
785	790	795
Val Ile Met Glu Leu Leu Ser Val Ala Ser Pro Asp Gly Ala Ser Leu		
805	810	815
Glu Lys Gln Gln Met Ile Val Arg Glu Lys Thr Gln Lys His Leu Asp		
820	825	830

-continued

Asp Tyr Ala Lys Gln Gly Leu Arg Thr Leu Cys Ile Ala Lys Lys Val
 835 840 845
 Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 850 855 860
 Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Glu Ser Ala Met
 865 870 875 880
 Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 885 890 895
 Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 900 905 910
 Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 915 920 925
 Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 930 935 940
 Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
 945 950 955 960
 Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
 965 970 975
 Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
 980 985 990
 Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
 995 1000 1005
 Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
 1010 1015 1020
 Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
 1025 1030 1035 1040
 Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Glu
 1045 1050 1055

<210> SEQ ID NO 7
 <211> LENGTH: 627
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 7

atgagtgcac ctgaatatgc agagtggctg aggaatcatt ttttagctga aaccagcatt 60
 gacaacaggg aagaattact acttgaatct gccatgaggt tggagaacaa acttacatta 120
 cttggtgcta ctggcattga agaccgtctg caggagggag tccttgaatc tatagaagct 180
 cttcacaag cgggcatcaa gatctggatg ctgacagggg acaagcagga gacagctgtc 240
 aacatagctt atgcatgcaa actactggag ccagatgaca agctttttat cctcaatacc 300
 caaagtaaa atgcctgtgg gatgctgatg agcacaattt tgaagaact tcagaagaaa 360
 actcaagccc tgccagagca agtgtcatta agtgaagatt tacttcagcc tcctgtcccc 420
 cgggactcag ggttacgagc tggactcatt atcactggga agaccctgga gtttgccctg 480
 caagaaagtc tgcaaaagca gttcctggaa ctgacatctt ggtgtcaagc tgtggtctgc 540
 tgccgagcca caccgctgca gaaaagtga gtggtgaaat tgggtccgag ccatctccag 600
 gtgatgaccc ttgctattgg tgagtga 627

<210> SEQ ID NO 8
 <211> LENGTH: 208
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 8

```

Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 1         5         10        15
Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Glu Ser Ala Met
 20        25        30
Arg Leu Glu Asn Lys Leu Thr Leu Gly Ala Thr Gly Ile Glu Asp
 35        40        45
Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 50        55        60
Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 65        70        75        80
Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 85        90        95
Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
100       105       110
Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
115       120       125
Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
130       135       140
Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
145       150       155       160
Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
165       170       175
Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
180       185       190
Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Glu
195       200       205

```

<210> SEQ ID NO 9

<211> LENGTH: 3813

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 9

```

atgactgagg ctctccaatg ggccagatat cactggcgac ggctgatcag aggtgcaacc      60
agggatgatg attcagggcc atacaactat tcctcgttgc tcgcctgtgg gcgcaagtcc      120
tctcagatcc ctaaaactgc aggaaggcac cggattgttg ttccccacat ccagcccttc      180
aaggatgagt atgagaagtt ctccggagcc tatgtgaaca atcgaatacg aacaacaaag      240
tacacacttc tgaattttgt gccaagaaat ttatttgaac aatttcacag agctgccaat      300
ttatatttcc tgttcctagt tgtcctgaac tgggtacctt tggtagaagc cttccaaaag      360
gaaatcacca tgttgctctc ggtggtggtc cttacaatta tcgcaattaa agatggcctg      420
gaagattatc ggaataacaa aattgacaaa cagatcaata atttaataac taaagtttat      480
agtaggaaag agaaaaaata cattgaccga tgctggaaag acgttactgt tggggacttt      540
attcgctctc cctgcaacga ggtcatccct gcagacatgg tactactctt ttccactgat      600
ccagatggaa tctgtcacat tgagacttct ggtcttgatg gagagagcaa tttaaaacag      660
aggcaggttg ttccggggata tgcagaacag gactctgaag ttgatcctga gaagttttcc      720
agtaggatag aatgtgaaag cccaaacaat gacctcagca gattccgagg cttcctagaa      780
cattccaaca aagaacgcgt gggctctcagt aaagaaaatt tgttgcttag aggatgcacc      840
attagaanca cagaggctgt tgtgggcatt gtggtttatg caggccatga aaccaaagca      900

```

-continued

atgctgaaca	acagtgggcc	acggtataag	cgcagcaaat	tagaaagaag	agcaaacaca	960
gatgtcctct	gggtgtgcat	gcttctggtc	ataatgtgct	taactggcgc	agtaggtcat	1020
ggaatctggc	tgagcaggta	tgaagagatg	cattttttca	atgttcccg	gcctgatgga	1080
catatcatat	caccactgtt	ggcaggatgt	tatatgtttt	ggacatgat	cattttgtta	1140
caggctctga	ttcctatttc	tctctatggt	tccatcgaaa	ttgtgaagct	tggaacaata	1200
tatttcattc	aaagtgtagt	ggattttctac	aatgaaaaaa	tggttcttat	tgttcagtgc	1260
cgagccctga	acatcgccga	ggatctggga	cagattcagt	acctcttttc	cgataagaca	1320
ggaaccctca	ctgagaataa	gatggttttt	cgaagatgta	gtgtggcagg	atttgattac	1380
tgccatgaag	aaaatgccag	gaggttgagg	tcctatcagg	aagctgtctc	tgaagatgaa	1440
gattttatag	acacagtcag	tggttccctc	agcaatatgg	caaaaccgag	agcccccagc	1500
tgacggacag	ttcataatgg	gcctttggga	aataagccct	caaatcatct	tgctggggagc	1560
tcttttactc	taggaagtgg	agaaggagcc	agtgaagtgc	ctcattccag	acaggctgct	1620
ttcagtagcc	ccattgaaac	agacgtggta	ccagacacca	ggctttttaga	caaatttagt	1680
cagattacac	ctcggtctct	tatgccacta	gatgagacca	tccaaaatcc	accaatggaa	1740
actttgtaca	ttatcgactt	tttcattgca	ttggcaattt	gcaacacagt	agtggtttct	1800
gctcctaacc	aaccccgaca	aaagatcaga	cacccttcac	tggtgggggtt	gccatttaag	1860
tctttggaag	agattaaaag	tcttttccag	agatgggtctg	tccgaagatc	aagttctcca	1920
tcgcttaaca	gtgggaaga	gccatcttct	ggagttccaa	acgcctttgt	gagcagactc	1980
cctctcttta	gtcgaatgaa	accagcttca	cctgtggagg	aagaggtctc	ccagggtgtgt	2040
gagagccccc	agtgtccag	tagctcagct	tgctgcacag	aaacagagaa	acaacacggt	2100
gatgcaggcc	tcctgaatgg	caaggcagag	tccctccctg	gacagccatt	ggcctgcaac	2160
ctgtgttatg	aggccgagag	cccagacgaa	gcggccttag	tgtatgccgc	caggggttac	2220
caatgcactt	tacggtctcg	gacaccagag	caggtcatgg	tggaacttgc	tgctttggga	2280
ccattaacat	ttcaactcct	acacatcctg	ccctttgact	cagtaagaaa	agaatgtct	2340
gttgtggtcc	gacaccctct	ttccaatcaa	gttgtggtgt	atcgaaaagg	cgctgattct	2400
gtgatcatgg	agttactgtc	ggtggcttcc	ccagatggag	caagtctgga	gaaacaacag	2460
atgatagtaa	gggagaaaac	ccagaagcac	ttggatgact	atgccaaaca	aggccttcgt	2520
actttatgta	tagcaagaaa	ggtcatgagt	gacactgaat	atgcagagtg	gctgaggaat	2580
catttttttag	ctgaaaccag	cattgacaac	agggaagaat	tactacttga	atctgccatg	2640
agggtggaga	acaaacttac	attacttgg	gctactggca	ttgaagaccg	tctgcaggag	2700
ggagtccctg	aatctataga	agctcttcac	aaagcgggca	tcaagatctg	gatgtgaca	2760
ggggacaagc	aggagacagc	tgtcaacata	gcttatgcat	gcaactact	ggagccagat	2820
gacaagcttt	ttatcctcaa	tacccaaagt	aaagatgcct	gtgggatgct	gatgagcaca	2880
attttgaaa	aacttcagaa	gaaaactcaa	gccctgccag	agcaagtgtc	attaagttaa	2940
gattttactc	agcctcctgt	ccccggggac	tcagggttac	gagctggact	cattatcact	3000
gggaagaccc	tggaagtttc	cctgcaagaa	agtctgcaaa	agcagttcct	ggaactgaca	3060
tcttggtgtc	aagctgtggt	ctgtgcccga	gccacaccgc	tcagaaaaag	tgaagtgtgt	3120
aaattggctc	gcagccatct	ccagggtgat	acccttgcta	ttggtgatgg	tgccaatgat	3180
gttagcatga	tacaagtggc	agacattggg	ataggggtct	caggtcaaga	aggcatgcag	3240
gctgtgatgg	ccagtgaact	tgccgtttct	cagttcaaac	atctcagcaa	gctccttctt	3300

-continued

```

gtccatggac actggtgta tacacggctt tccaacatga ttctctattt ttctataag 3360
aatgtggcct atgtgaacct ccttttctgg taccagttct tttgtggatt ttcaggaaca 3420
tccatgactg attactgggt ttgatcttc ttcaacctcc tcttcacatc tgcccctcct 3480
gtcatttatg gtgttttga gaaagatgtg tctgcagaga ccctcatgca actgcctgaa 3540
ctttacagaa gtggtcagaa atcagaggca tacttaccoc atacctctg gatcacctta 3600
ttggatgctt tttatcaaag cctgggtctgc ttctttgtgc cttattttac ctaccagggc 3660
tcagatactg acatctttgc atttgaaac ccctgaaca cagccactct gttcatcggt 3720
ctcctccatc tggtcattga aagcaagagt ttgaccaggt gcagtgactc acacctgcaa 3780
ttccagagct ttgggaggct gtggatcaca tga 3813

```

```

<210> SEQ ID NO 10
<211> LENGTH: 1270
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 10

```

```

Met Thr Glu Ala Leu Gln Trp Ala Arg Tyr His Trp Arg Arg Leu Ile
 1             5             10             15
Arg Gly Ala Thr Arg Asp Asp Asp Ser Gly Pro Tyr Asn Tyr Ser Ser
          20             25             30
Leu Leu Ala Cys Gly Arg Lys Ser Ser Gln Ile Pro Lys Leu Ser Gly
          35             40             45
Arg His Arg Ile Val Val Pro His Ile Gln Pro Phe Lys Asp Glu Tyr
          50             55             60
Glu Lys Phe Ser Gly Ala Tyr Val Asn Asn Arg Ile Arg Thr Thr Lys
65             70             75             80
Tyr Thr Leu Leu Asn Phe Val Pro Arg Asn Leu Phe Glu Gln Phe His
          85             90             95
Arg Ala Ala Asn Leu Tyr Phe Leu Phe Leu Val Val Leu Asn Trp Val
          100            105            110
Pro Leu Val Glu Ala Phe Gln Lys Glu Ile Thr Met Leu Pro Leu Val
          115            120            125
Val Val Leu Thr Ile Ile Ala Ile Lys Asp Gly Leu Glu Asp Tyr Arg
          130            135            140
Lys Tyr Lys Ile Asp Lys Gln Ile Asn Asn Leu Ile Thr Lys Val Tyr
145            150            155            160
Ser Arg Lys Glu Lys Lys Tyr Ile Asp Arg Cys Trp Lys Asp Val Thr
          165            170            175
Val Gly Asp Phe Ile Arg Leu Ser Cys Asn Glu Val Ile Pro Ala Asp
          180            185            190
Met Val Leu Leu Phe Ser Thr Asp Pro Asp Gly Ile Cys His Ile Glu
          195            200            205
Thr Ser Gly Leu Asp Gly Glu Ser Asn Leu Lys Gln Arg Gln Val Val
          210            215            220
Arg Gly Tyr Ala Glu Gln Asp Ser Glu Val Asp Pro Glu Lys Phe Ser
225            230            235            240
Ser Arg Ile Glu Cys Glu Ser Pro Asn Asn Asp Leu Ser Arg Phe Arg
          245            250            255
Gly Phe Leu Glu His Ser Asn Lys Glu Arg Val Gly Leu Ser Lys Glu
          260            265            270
Asn Leu Leu Leu Arg Gly Cys Thr Ile Arg Asn Thr Glu Ala Val Val

```

-continued

275	280	285
Gly Ile Val Val Tyr Ala	Gly His Glu Thr Lys	Ala Met Leu Asn Asn
290	295	300
Ser Gly Pro Arg Tyr Lys	Arg Ser Lys Leu Glu	Arg Arg Ala Asn Thr
305	310	315
Asp Val Leu Trp Cys Val	Met Leu Leu Val Ile	Met Cys Leu Thr Gly
325	330	335
Ala Val Gly His Gly Ile	Trp Leu Ser Arg Tyr	Glu Lys Met His Phe
340	345	350
Phe Asn Val Pro Glu Pro	Asp Gly His Ile Ile	Ser Pro Leu Leu Ala
355	360	365
Gly Phe Tyr Met Phe Trp	Thr Met Ile Ile Leu	Leu Gln Val Leu Ile
370	375	380
Pro Ile Ser Leu Tyr Val	Ser Ile Glu Ile Val	Lys Leu Gly Gln Ile
385	390	395
Tyr Phe Ile Gln Ser Asp	Val Asp Phe Tyr Asn	Glu Lys Met Asp Ser
405	410	415
Ile Val Gln Cys Arg Ala	Leu Asn Ile Ala Glu	Asp Leu Gly Gln Ile
420	425	430
Gln Tyr Leu Phe Ser Asp	Lys Thr Gly Thr Leu	Thr Glu Asn Lys Met
435	440	445
Val Phe Arg Arg Cys Ser	Val Ala Gly Phe Asp	Tyr Cys His Glu Glu
450	455	460
Asn Ala Arg Arg Leu Glu	Ser Tyr Gln Glu Ala	Val Ser Glu Asp Glu
465	470	475
Asp Phe Ile Asp Thr Val	Ser Gly Ser Leu Ser	Asn Met Ala Lys Pro
485	490	495
Arg Ala Pro Ser Cys Arg	Thr Val His Asn Gly	Pro Leu Gly Asn Lys
500	505	510
Pro Ser Asn His Leu Ala	Gly Ser Ser Phe Thr	Leu Gly Ser Gly Glu
515	520	525
Gly Ala Ser Glu Val Pro	His Ser Arg Gln Ala	Ala Phe Ser Ser Pro
530	535	540
Ile Glu Thr Asp Val Val	Pro Asp Thr Arg Leu	Leu Asp Lys Phe Ser
545	550	555
Gln Ile Thr Pro Arg Leu	Phe Met Pro Leu Asp	Glu Thr Ile Gln Asn
565	570	575
Pro Pro Met Glu Thr Leu	Tyr Ile Ile Asp Phe	Phe Ile Ala Leu Ala
580	585	590
Ile Cys Asn Thr Val Val	Val Ser Ala Pro Asn	Gln Pro Arg Gln Lys
595	600	605
Ile Arg His Pro Ser Leu	Gly Gly Leu Pro Ile	Lys Ser Leu Glu Glu
610	615	620
Ile Lys Ser Leu Phe Gln	Arg Trp Ser Val Arg	Arg Ser Ser Ser Pro
625	630	635
Ser Leu Asn Ser Gly Lys	Glu Pro Ser Ser Gly	Val Pro Asn Ala Phe
645	650	655
Val Ser Arg Leu Pro Leu	Phe Ser Arg Met Lys	Pro Ala Ser Pro Val
660	665	670
Glu Glu Glu Val Ser Gln	Val Cys Glu Ser Pro	Gln Cys Ser Ser Ser
675	680	685
Ser Ala Cys Cys Thr Glu	Thr Glu Lys Gln His	Gly Asp Ala Gly Leu
690	695	700

-continued

Leu Asn Gly Lys Ala Glu Ser Leu Pro Gly Gln Pro Leu Ala Cys Asn
 705 710 715 720
 Leu Cys Tyr Glu Ala Glu Ser Pro Asp Glu Ala Ala Leu Val Tyr Ala
 725 730 735
 Ala Arg Ala Tyr Gln Cys Thr Leu Arg Ser Arg Thr Pro Glu Gln Val
 740 745 750
 Met Val Asp Phe Ala Ala Leu Gly Pro Leu Thr Phe Gln Leu Leu His
 755 760 765
 Ile Leu Pro Phe Asp Ser Val Arg Lys Arg Met Ser Val Val Val Arg
 770 775 780
 His Pro Leu Ser Asn Gln Val Val Val Tyr Thr Lys Gly Ala Asp Ser
 785 790 795 800
 Val Ile Met Glu Leu Leu Ser Val Ala Ser Pro Asp Gly Ala Ser Leu
 805 810 815
 Glu Lys Gln Gln Met Ile Val Arg Glu Lys Thr Gln Lys His Leu Asp
 820 825 830
 Asp Tyr Ala Lys Gln Gly Leu Arg Thr Leu Cys Ile Ala Lys Lys Val
 835 840 845
 Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 850 855 860
 Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
 865 870 875 880
 Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 885 890 895
 Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 900 905 910
 Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 915 920 925
 Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 930 935 940
 Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
 945 950 955 960
 Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
 965 970 975
 Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
 980 985 990
 Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
 995 1000 1005
 Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
 1010 1015 1020
 Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
 1025 1030 1035 1040
 Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Asp
 1045 1050 1055
 Gly Ala Asn Asp Val Ser Met Ile Gln Val Ala Asp Ile Gly Ile Gly
 1060 1065 1070
 Val Ser Gly Gln Glu Gly Met Gln Ala Val Met Ala Ser Asp Phe Ala
 1075 1080 1085
 Val Ser Gln Phe Lys His Leu Ser Lys Leu Leu Leu Val His Gly His
 1090 1095 1100
 Trp Cys Tyr Thr Arg Leu Ser Asn Met Ile Leu Tyr Phe Phe Tyr Lys
 1105 1110 1115 1120

-continued

Asn Val Ala Tyr Val Asn Leu Leu Phe Trp Tyr Gln Phe Phe Cys Gly
 1125 1130 1135
 Phe Ser Gly Thr Ser Met Thr Asp Tyr Trp Val Leu Ile Phe Phe Asn
 1140 1145 1150
 Leu Leu Phe Thr Ser Ala Pro Pro Val Ile Tyr Gly Val Leu Glu Lys
 1155 1160 1165
 Asp Val Ser Ala Glu Thr Leu Met Gln Leu Pro Glu Leu Tyr Arg Ser
 1170 1175 1180
 Gly Gln Lys Ser Glu Ala Tyr Leu Pro His Thr Phe Trp Ile Thr Leu
 1185 1190 1195 1200
 Leu Asp Ala Phe Tyr Gln Ser Leu Val Cys Phe Phe Val Pro Tyr Phe
 1205 1210 1215
 Thr Tyr Gln Gly Ser Asp Thr Asp Ile Phe Ala Phe Gly Asn Pro Leu
 1220 1225 1230
 Asn Thr Ala Thr Leu Phe Ile Val Leu Leu His Leu Val Ile Glu Ser
 1235 1240 1245
 Lys Ser Leu Thr Arg Cys Ser Asp Ser His Leu Gln Phe Gln Ser Phe
 1250 1255 1260
 Gly Arg Leu Trp Ile Thr
 1265 1270

<210> SEQ ID NO 11
 <211> LENGTH: 1269
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 11

atgagtgcac ctgaatatgc agagtggctg aggaatcatt ttttagctga aaccagcatt 60
 gacaacaggg aagaattact acttgaatct gccatgaggt tggagaacaa acttacatta 120
 cttggtgcta ctggcattga agaccgtctg caggagggag tccctgaatc tatagaagct 180
 cttcacaaag cgggcatcaa gatctggatg ctgacagggg acaagcagga gacagctgtc 240
 aacatagctt atgcatgcaa actactggag ccagatgaca agctttttat cctcaatacc 300
 caaagtaaag atgcctgtgg gatgctgatg agcacaattt tgaaagaact tcagaagaaa 360
 actcaagccc tgccagagca agtgtcatta agtgaagatt tacttcagcc tcctgtcccc 420
 cgggactcag ggttacgagc tggactcatt atcactggga agaccctgga gtttgccctg 480
 caagaaagtc tgcaaaagca gttcctggaa ctgacatctt ggtgtcaagc tgtggtctgc 540
 tgccgagcca caccgtgca gaaaagtga gttgtgaaat tgggtccgag ccatctccag 600
 gtgatgaccc ttgctattgg tgatgggtgcc aatgatgtta gcatgataca agtggcagac 660
 attgggatag gggctctcag tcaagaaggc atgcaggctg tgatggccag tgactttgcc 720
 gtttctcagt tcaaacatct cagcaagctc cttcttgtcc atggacactg gtgttatata 780
 cggcctttcca acatgattct ctattttttc tataagaatg tggcctatgt gaacctcctt 840
 ttctgggtacc agttcttttg tggattttca ggaacatcca tgactgatta ctgggttttg 900
 atctttttca acctcctctt cacatctgcc cctcctgtca tttatgggtg tttggagaaa 960
 gatgtgtctg cagagaccct catgcaactg cctgaacttt acagaagtgg tcagaaatca 1020
 gaggcatact taccocatcc cttctggatc accttattgg atgcttttta tcaaagcctg 1080
 gtctgcttct ttgtgcctta tttacctac cagggtctag atactgacat ctttgcatct 1140
 ggaaaccccc tgaacacagc cactctgttc atcgtttctc tccatctggt cattgaaagc 1200
 aagagtttga ccagggtcag tgactcacac ctgcaattcc agagcttttg gaggtgtggt 1260

-continued

atcacatga

1269

<210> SEQ ID NO 12
 <211> LENGTH: 422
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 12

```

Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 1             5             10            15

Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
      20             25            30

Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 35             40            45

Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 50             55            60

Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 65             70            75            80

Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 85             90            95

Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
100            105            110

Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
115            120            125

Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
130            135            140

Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
145            150            155            160

Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
165            170            175

Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
180            185            190

Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Asp
195            200            205

Gly Ala Asn Asp Val Ser Met Ile Gln Val Ala Asp Ile Gly Ile Gly
210            215            220

Val Ser Gly Gln Glu Gly Met Gln Ala Val Met Ala Ser Asp Phe Ala
225            230            235            240

Val Ser Gln Phe Lys His Leu Ser Lys Leu Leu Val His Gly His
245            250            255

Trp Cys Tyr Thr Arg Leu Ser Asn Met Ile Leu Tyr Phe Phe Tyr Lys
260            265            270

Asn Val Ala Tyr Val Asn Leu Leu Phe Trp Tyr Gln Phe Phe Cys Gly
275            280            285

Phe Ser Gly Thr Ser Met Thr Asp Tyr Trp Val Leu Ile Phe Phe Asn
290            295            300

Leu Leu Phe Thr Ser Ala Pro Pro Val Ile Tyr Gly Val Leu Glu Lys
305            310            315            320

Asp Val Ser Ala Glu Thr Leu Met Gln Leu Pro Glu Leu Tyr Arg Ser
325            330            335

Gly Gln Lys Ser Glu Ala Tyr Leu Pro His Thr Phe Trp Ile Thr Leu
340            345            350

Leu Asp Ala Phe Tyr Gln Ser Leu Val Cys Phe Phe Val Pro Tyr Phe
355            360            365

```

-continued

Thr Tyr Gln Gly Ser Asp Thr Asp Ile Phe Ala Phe Gly Asn Pro Leu
 370 375 380
 Asn Thr Ala Thr Leu Phe Ile Val Leu Leu His Leu Val Ile Glu Ser
 385 390 395 400
 Lys Ser Leu Thr Arg Cys Ser Asp Ser His Leu Gln Phe Gln Ser Phe
 405 410 415
 Gly Arg Leu Trp Ile Thr
 420

<210> SEQ ID NO 13
 <211> LENGTH: 4281
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 13

atgactgagg ctctccaatg ggccagatat cactggcgac ggctgatcag aggtgcaacc 60
 agggatgatg attcagggcc atacaactat tcctcgttgc tcgctgtggy ggcgaagtcc 120
 tctcagatcc cttaactgtc aggaaggcac cggattgttg ttccccacat ccagcccttc 180
 aaggatgagt atgagaagtt ctccggagcc tatgtgaaca atcgaatacy aacaacaaag 240
 tacacacttc tgaattttgt gccaaagaaat ttatttgaac aatttcacag agctgccaat 300
 ttatatattcc tgttcctagt tgtcctgaac tgggtacctt tggtagaagc cttccaaaag 360
 gaaatcacca tgttgctctt ggtgggtggc cttacaatta tcgcaattaa agatggcctg 420
 gaagattatc ggaatacaaa aattgacaaa cagatcaata atttaataac taaagtttat 480
 agtaggaaa agaaaaata cattgaccga tgcctggaag acgttactgt tggggacttt 540
 attcgcctct cctgcaacga ggtcatccct gcagacatgg tactactctt ttccactgat 600
 ccagatggaa tctgtcacat tgagacttct ggtcttgatg gagagagcaa tttaaaacag 660
 aggcagggtg ttcggggata tgcagaacag gactctgaag ttgatcctga gaagttttcc 720
 agtaggatag aatgtgaaag cccaaacaat gacctcagca gattccgagg ctctcctagaa 780
 cattccaaca aagaacgcgt gggctcagc aaagaaaatt tgttgcttag aggatgcacc 840
 attagaaaca cagaggctgt tgtgggcatt gtggtttatg caggccatga aaccaaagca 900
 atgctgaaca acagtgggco acgttataag cgcagcaaat tagaaagaag agcaaacaca 960
 gatgtcctct ggtgtgtcat gcttctggtc ataattgtct taactggcgc agtaggtcat 1020
 ggaatctgcy tgagcaggta tgaagagatg cattttttca atgttcccga gcctgatgga 1080
 catatcatat caccactgtt ggcaggattt tatatgtttt ggaccatgat cattttgtta 1140
 caggctctga ttcctatttc tctctatgtt tccatcgaaa ttgtgaagct tggacaaata 1200
 tattttcatt aaagtgatgt ggatttctac aatgaaaaaa tggattctat tgttcagtgc 1260
 cgagccctga acatgcgcga ggaatctgga cagattcagt acctcttttc cgataagaca 1320
 ggaaccctca ctgagaataa gatgggtttt cgaagatgta gtgtggcagg atttgattac 1380
 tgccatgaag aaatgcccga gaggttgag tcctatcagg aagctgtctc tgaagatgaa 1440
 gattttatag acacagtcag tggttccctc agcaaatatg caaaaccgag agccccagc 1500
 tgcaggacag ttcataatgg gcctttggga aataagccct caaatcatct tctgtgggagc 1560
 tcttttactc taggaagtgg agaaggagcc agtgaagtgc ctcatccag acaggctgct 1620
 ttcagtagcc ccattgaac agacgtggta ccagacacca ggcttttaga caaatttagt 1680
 cagattacac ctgcggtctt tatgccacta gatgagacca tccaaaatcc accaatggaa 1740

-continued

actttgtaca ttatcgactt tttcattgca ttggcaattt gcaacacagt agtggtttct	1800
gctcctaacc aacccccaca aaagatcaga cacccttcac tgggggggtt gccattaag	1860
tctttggaag agattaaaag tcttttccag agatggctctg tccgaagatc aagttctcca	1920
tcgcttaaca gtgggaaaga gccatcttct ggagttccaa acgcctttgt gagcagactc	1980
cctctcttta gtcgaaatga accagcttca cctgtggagg aagaggtctc ccaggtgtgt	2040
gagagccccc agtgctccag tagctcagct tgctgcacag aaacagagaa acaacacggt	2100
gatgcaggcc tcctgaatgg caaggcagag tccctccctg gacagccatt ggcctgcaac	2160
ctgtgttatg aggccgagag cccagacgaa gcggccttag tgtatgccgc cagggtttac	2220
caatgcactt tacggtctcg gacaccagag caggtcatgg tggactttgc tgccttggga	2280
ccattaacat ttcaactcct acacatcctg ccctttgact cagtaagaaa aagaatgtct	2340
gttgtgttcc gacaccctct ttccaatcaa gttgtggtgt atacgaaagg cgctgattct	2400
gtgatcatgg agttactgtc ggtggcttcc ccagatggag caagtctgga gaaacaacag	2460
atgatatgtaa gggagaaaac ccagaagcac ttggatgact atgccaaaca aggccttcgt	2520
actttatgta tagcaagaa ggtcatgagt gacactgaat atgcagagtg gctgaggaat	2580
cattttttat ctgaaaccag cattgacaac agggaagaat tactacttga atctgccatg	2640
aggttggaga acaaaacttac attacttggg gctactggca ttgaagaccg tctgcaggag	2700
ggagtccctg aatctataga agctcttcac aaagcgggca tcaagatctg gatgctgaca	2760
ggggacaacg aggagacagc tgtcaacata gcttatgcat gcaaaactact ggagccagat	2820
gacaagcttt ttatcctcaa tacccaaagt aaagatgcct gtgggatgct gatgagcaca	2880
attttgaag aacttcagaa gaaaactcaa gccctgccag agcaagtgtc attaagtga	2940
gatttacttc agcctcctgt ccccccggac tcagggttac gagctggact cattatcaat	3000
gggaagaccc tggagtttgc cctgcaagaa agtctgcaa agcagttcct ggaactgaca	3060
tcttggtgtc aagctgtggt ctgctgccga gccacaccgc tgcagaaaag tgaagtgtg	3120
aaattggtcc gcagccatct ccaggtgatg acccttgcta ttggtgatgg tgccaatgat	3180
gttagcatga tacaagtggc agacattggg ataggggtct caggtcaaga aggcattgag	3240
gctgtgatgg ccagtgaact tgccgtttct cagttcaaac atctcagcaa gctccttctt	3300
gtccatggac actggtgtta tacacggcct tccaacatga ttctctattt ttctataag	3360
aatgtggcct atgtgaacct ccttttctg taccagttct ttgtggatt ttcaggaaaca	3420
tccatgactg attactgggt tttgatcttc ttcaacctcc tcttcacatc tgccccctct	3480
gtcatttatg gtgttttggg gaaagatgtg tctgcagaga cctcatgca actgcctgaa	3540
ctttacagaa gtggtcagaa atcagaggca tacttacctc atacctctg gatcacctta	3600
ttggatgctt ttatcaaa cctggtctgc ttctttgtgc cttattttac ctaccagggc	3660
tcagatactg acatctttgc atttggaac cccctgaaca cagccactct gttcatcggt	3720
ctcctccatc tggtcattga aagcaagagt ttgacttggg ttcacttgct ggtcatcatt	3780
ggtagcatct tgtcttattt ttattttgcc atagtttttg gagccatgtg tgtaacttgc	3840
aaccaccat ccaacctta ctggattatg caggagcaca tgctggatcc agtattctac	3900
ttagtttgta tcctcacgac gtccattgct cttctgcca ggtttgtata cagagttctt	3960
cagggatccc tgtttccatc tccaattctg agagctaagc actttgacag actaactcca	4020
gaggagagga ctaaaactct caagaagtgg agaggggctg gaaagatgaa tcaagtgaca	4080
tcaaagtatg ctaaccaatc agctggcaag tcaggaagaa gacctatgcc tggcccttct	4140

-continued

gctgtatttg caatgaagtc agcaacttcc tgtgctattg agcaaggaaa cttatctctg 4200
 tgtgaaactg ctttagatca aggctactct gaaactaagg cctttgagat ggctggaccc 4260
 tccaaaggta aagaaagcta g 4281

<210> SEQ ID NO 14
 <211> LENGTH: 1426
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens
 <400> SEQUENCE: 14

Met Thr Glu Ala Leu Gln Trp Ala Arg Tyr His Trp Arg Arg Leu Ile
 1 5 10 15
 Arg Gly Ala Thr Arg Asp Asp Asp Ser Gly Pro Tyr Asn Tyr Ser Ser
 20 25 30
 Leu Leu Ala Cys Gly Arg Lys Ser Ser Gln Ile Pro Lys Leu Ser Gly
 35 40 45
 Arg His Arg Ile Val Val Pro His Ile Gln Pro Phe Lys Asp Glu Tyr
 50 55 60
 Glu Lys Phe Ser Gly Ala Tyr Val Asn Asn Arg Ile Arg Thr Thr Lys
 65 70 75 80
 Tyr Thr Leu Leu Asn Phe Val Pro Arg Asn Leu Phe Glu Gln Phe His
 85 90 95
 Arg Ala Ala Asn Leu Tyr Phe Leu Phe Leu Val Val Leu Asn Trp Val
 100 105 110
 Pro Leu Val Glu Ala Phe Gln Lys Glu Ile Thr Met Leu Pro Leu Val
 115 120 125
 Val Val Leu Thr Ile Ile Ala Ile Lys Asp Gly Leu Glu Asp Tyr Arg
 130 135 140
 Lys Tyr Lys Ile Asp Lys Gln Ile Asn Asn Leu Ile Thr Lys Val Tyr
 145 150 155 160
 Ser Arg Lys Glu Lys Lys Tyr Ile Asp Arg Cys Trp Lys Asp Val Thr
 165 170 175
 Val Gly Asp Phe Ile Arg Leu Ser Cys Asn Glu Val Ile Pro Ala Asp
 180 185 190
 Met Val Leu Leu Phe Ser Thr Asp Pro Asp Gly Ile Cys His Ile Glu
 195 200 205
 Thr Ser Gly Leu Asp Gly Glu Ser Asn Leu Lys Gln Arg Gln Val Val
 210 215 220
 Arg Gly Tyr Ala Glu Gln Asp Ser Glu Val Asp Pro Glu Lys Phe Ser
 225 230 235 240
 Ser Arg Ile Glu Cys Glu Ser Pro Asn Asn Asp Leu Ser Arg Phe Arg
 245 250 255
 Gly Phe Leu Glu His Ser Asn Lys Glu Arg Val Gly Leu Ser Lys Glu
 260 265 270
 Asn Leu Leu Leu Arg Gly Cys Thr Ile Arg Asn Thr Glu Ala Val Val
 275 280 285
 Gly Ile Val Val Tyr Ala Gly His Glu Thr Lys Ala Met Leu Asn Asn
 290 295 300
 Ser Gly Pro Arg Tyr Lys Arg Ser Lys Leu Glu Arg Arg Ala Asn Thr
 305 310 315 320
 Asp Val Leu Trp Cys Val Met Leu Leu Val Ile Met Cys Leu Thr Gly
 325 330 335
 Ala Val Gly His Gly Ile Trp Leu Ser Arg Tyr Glu Lys Met His Phe

-continued

340	345	350
Phe Asn Val Pro Glu Pro Asp Gly His Ile Ile Ser Pro Leu Leu Ala 355 360 365		
Gly Phe Tyr Met Phe Trp Thr Met Ile Ile Leu Leu Gln Val Leu Ile 370 375 380		
Pro Ile Ser Leu Tyr Val Ser Ile Glu Ile Val Lys Leu Gly Gln Ile 385 390 395 400		
Tyr Phe Ile Gln Ser Asp Val Asp Phe Tyr Asn Glu Lys Met Asp Ser 405 410 415		
Ile Val Gln Cys Arg Ala Leu Asn Ile Ala Glu Asp Leu Gly Gln Ile 420 425 430		
Gln Tyr Leu Phe Ser Asp Lys Thr Gly Thr Leu Thr Glu Asn Lys Met 435 440 445		
Val Phe Arg Arg Cys Ser Val Ala Gly Phe Asp Tyr Cys His Glu Glu 450 455 460		
Asn Ala Arg Arg Leu Glu Ser Tyr Gln Glu Ala Val Ser Glu Asp Glu 465 470 475 480		
Asp Phe Ile Asp Thr Val Ser Gly Ser Leu Ser Asn Met Ala Lys Pro 485 490 495		
Arg Ala Pro Ser Cys Arg Thr Val His Asn Gly Pro Leu Gly Asn Lys 500 505 510		
Pro Ser Asn His Leu Ala Gly Ser Ser Phe Thr Leu Gly Ser Gly Glu 515 520 525		
Gly Ala Ser Glu Val Pro His Ser Arg Gln Ala Ala Phe Ser Ser Pro 530 535 540		
Ile Glu Thr Asp Val Val Pro Asp Thr Arg Leu Leu Asp Lys Phe Ser 545 550 555 560		
Gln Ile Thr Pro Arg Leu Phe Met Pro Leu Asp Glu Thr Ile Gln Asn 565 570 575		
Pro Pro Met Glu Thr Leu Tyr Ile Ile Asp Phe Phe Ile Ala Leu Ala 580 585 590		
Ile Cys Asn Thr Val Val Val Ser Ala Pro Asn Gln Pro Arg Gln Lys 595 600 605		
Ile Arg His Pro Ser Leu Gly Gly Leu Pro Ile Lys Ser Leu Glu Glu 610 615 620		
Ile Lys Ser Leu Phe Gln Arg Trp Ser Val Arg Arg Ser Ser Ser Pro 625 630 635 640		
Ser Leu Asn Ser Gly Lys Glu Pro Ser Ser Gly Val Pro Asn Ala Phe 645 650 655		
Val Ser Arg Leu Pro Leu Phe Ser Arg Met Lys Pro Ala Ser Pro Val 660 665 670		
Glu Glu Glu Val Ser Gln Val Cys Glu Ser Pro Gln Cys Ser Ser Ser 675 680 685		
Ser Ala Cys Cys Thr Glu Thr Glu Lys Gln His Gly Asp Ala Gly Leu 690 695 700		
Leu Asn Gly Lys Ala Glu Ser Leu Pro Gly Gln Pro Leu Ala Cys Asn 705 710 715 720		
Leu Cys Tyr Glu Ala Glu Ser Pro Asp Glu Ala Ala Leu Val Tyr Ala 725 730 735		
Ala Arg Ala Tyr Gln Cys Thr Leu Arg Ser Arg Thr Pro Glu Gln Val 740 745 750		
Met Val Asp Phe Ala Ala Leu Gly Pro Leu Thr Phe Gln Leu Leu His 755 760 765		

-continued

Ile Leu Pro Phe Asp Ser Val Arg Lys Arg Met Ser Val Val Val Arg
 770 775 780
 His Pro Leu Ser Asn Gln Val Val Val Tyr Thr Lys Gly Ala Asp Ser
 785 790 795 800
 Val Ile Met Glu Leu Leu Ser Val Ala Ser Pro Asp Gly Ala Ser Leu
 805 810 815
 Glu Lys Gln Gln Met Ile Val Arg Glu Lys Thr Gln Lys His Leu Asp
 820 825 830
 Asp Tyr Ala Lys Gln Gly Leu Arg Thr Leu Cys Ile Ala Lys Lys Val
 835 840 845
 Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 850 855 860
 Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
 865 870 875 880
 Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
 885 890 895
 Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
 900 905 910
 Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
 915 920 925
 Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
 930 935 940
 Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
 945 950 955 960
 Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
 965 970 975
 Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
 980 985 990
 Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
 995 1000 1005
 Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
 1010 1015 1020
 Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
 1025 1030 1035 1040
 Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Asp
 1045 1050 1055
 Gly Ala Asn Asp Val Ser Met Ile Gln Val Ala Asp Ile Gly Ile Gly
 1060 1065 1070
 Val Ser Gly Gln Glu Gly Met Gln Ala Val Met Ala Ser Asp Phe Ala
 1075 1080 1085
 Val Ser Gln Phe Lys His Leu Ser Lys Leu Leu Val His Gly His
 1090 1095 1100
 Trp Cys Tyr Thr Arg Leu Ser Asn Met Ile Leu Tyr Phe Phe Tyr Lys
 1105 1110 1115 1120
 Asn Val Ala Tyr Val Asn Leu Leu Phe Trp Tyr Gln Phe Phe Cys Gly
 1125 1130 1135
 Phe Ser Gly Thr Ser Met Thr Asp Tyr Trp Val Leu Ile Phe Phe Asn
 1140 1145 1150
 Leu Leu Phe Thr Ser Ala Pro Pro Val Ile Tyr Gly Val Leu Glu Lys
 1155 1160 1165
 Asp Val Ser Ala Glu Thr Leu Met Gln Leu Pro Glu Leu Tyr Arg Ser
 1170 1175 1180

-continued

Gly Gln Lys Ser Glu Ala Tyr Leu Pro His Thr Phe Trp Ile Thr Leu
 1185 1190 1195 1200
 Leu Asp Ala Phe Tyr Gln Ser Leu Val Cys Phe Phe Val Pro Tyr Phe
 1205 1210 1215
 Thr Tyr Gln Gly Ser Asp Thr Asp Ile Phe Ala Phe Gly Asn Pro Leu
 1220 1225 1230
 Asn Thr Ala Thr Leu Phe Ile Val Leu Leu His Leu Val Ile Glu Ser
 1235 1240 1245
 Lys Ser Leu Thr Trp Ile His Leu Leu Val Ile Ile Gly Ser Ile Leu
 1250 1255 1260
 Ser Tyr Phe Leu Phe Ala Ile Val Phe Gly Ala Met Cys Val Thr Cys
 1265 1270 1275 1280
 Asn Pro Pro Ser Asn Pro Tyr Trp Ile Met Gln Glu His Met Leu Asp
 1285 1290 1295
 Pro Val Phe Tyr Leu Val Cys Ile Leu Thr Thr Ser Ile Ala Leu Leu
 1300 1305 1310
 Pro Arg Phe Val Tyr Arg Val Leu Gln Gly Ser Leu Phe Pro Ser Pro
 1315 1320 1325
 Ile Leu Arg Ala Lys His Phe Asp Arg Leu Thr Pro Glu Glu Arg Thr
 1330 1335 1340
 Lys Ala Leu Lys Lys Trp Arg Gly Ala Gly Lys Met Asn Gln Val Thr
 1345 1350 1355 1360
 Ser Lys Tyr Ala Asn Gln Ser Ala Gly Lys Ser Gly Arg Arg Pro Met
 1365 1370 1375
 Pro Gly Pro Ser Ala Val Phe Ala Met Lys Ser Ala Thr Ser Cys Ala
 1380 1385 1390
 Ile Glu Gln Gly Asn Leu Ser Leu Cys Glu Thr Ala Leu Asp Gln Gly
 1395 1400 1405
 Tyr Ser Glu Thr Lys Ala Phe Glu Met Ala Gly Pro Ser Lys Gly Lys
 1410 1415 1420
 Glu Ser
 1425

<210> SEQ ID NO 15
 <211> LENGTH: 1737
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens
 <400> SEQUENCE: 15

atgagtgcac ctgaatatgc agagtggctg aggaatcatt ttttagctga aaccagcatt 60
 gacaacaggg aagaattact acttgaatct gccatgaggt tggagaacaa acttacatta 120
 cttggtgcta ctggcattga agaccgtctg caggaggagag tccctgaatc tatagaagct 180
 cttcaciaaag cgggcatcaa gatctggatg ctgacágggg acaagcagga gacagctgtc 240
 aacatagctt atgcatgcaa actactggag ccagatgaca agctttttat cctcaatacc 300
 caaagtaaaag atgcctgtgg gatgctgatg agcacaattt tgaagaact tcagaagaaa 360
 actcaagccc tgccagagca agtgtcatta agtgaagatt tacttcagcc tctgtccccc 420
 cgggactcag ggttacgagc tggactcatt atcactggga agaccctgga gttgcccctg 480
 caagaaagtc tgcaaaagca gttcctggaa ctgacatctt ggtgtcaagc tgtggtctgc 540
 tgccgagcca caccgctgca gaaaagtgaat gtggtgaaat tgggccgcag ccatctccag 600
 gtgatgaccc ttgctattgg tgatgggtgcc aatgatgtta gcatgataca agtggcagac 660
 attgggatag ggggtctcagg tcaagaaggc atgcaggctg tgatggccag tgactttgcc 720

-continued

```

gtttctcagt tcaaacatct cagcaagctc cttcttgtcc atggacactg gtgttataca 780
cggttttcca acatgattct ctattttttc tataagaatg tggcctatgt gaacctcctt 840
ttctggtacc agttcttttg tggattttca ggaacatcca tgactgatta ctggggtttg 900
atcttcttca acctcctctt cacatctgcc cctcctgtca tttatgggtg tttggagaaa 960
gatgtgtctg cagagaccct catgcaactg cctgaacttt acagaagtgg tcagaaatca 1020
gaggcatact taccctacac cttctggatc accttattgg atgcttttta tcaaaagctg 1080
gtctgcttct ttgtgcctta ttttacctac cagggtcag atactgacat ctttgcat 1140
ggaaaccccc tgaacacagc cactctgttc atcgttctcc tccatctggt cattgaaagc 1200
aagagtttga cttggattca cttgtgtgtc atcattggta gcactctgtc ttatttttta 1260
tttgccatag tttttggagc catgtgtgta acttgcaacc caccatccaa cccttactgg 1320
attatgcagg agcacatgct ggatccagta ttctacttag tttgtatcct cagcagctcc 1380
attgctcttc tgcccagggt tgtatacaga gttcttcagg gatccctggt tccatctcca 1440
attctgagag ctaagcactt tgacagacta actccagagg agaggactaa agctctcaag 1500
aagtggagag gggctggaaa gatgaatcaa gtgacatcaa agtatgctaa ccaatcagct 1560
ggcaagtcag gaagaagacc catgcctggc ccttctgtct tatttgcaat gaagtcagca 1620
acttctgtg ctattgagca aggaactta tctctgtgtg aaactgcttt agatcaaggc 1680
tactctgaaa ctaaggcctt tgagatggct ggaccctcca aaggtaaaga aagctag 1737

```

```

<210> SEQ ID NO 16
<211> LENGTH: 578
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 16

```

```

Met Ser Asp Thr Glu Tyr Ala Glu Trp Leu Arg Asn His Phe Leu Ala
 1             5             10             15
Glu Thr Ser Ile Asp Asn Arg Glu Glu Leu Leu Leu Glu Ser Ala Met
          20             25             30
Arg Leu Glu Asn Lys Leu Thr Leu Leu Gly Ala Thr Gly Ile Glu Asp
          35             40             45
Arg Leu Gln Glu Gly Val Pro Glu Ser Ile Glu Ala Leu His Lys Ala
          50             55             60
Gly Ile Lys Ile Trp Met Leu Thr Gly Asp Lys Gln Glu Thr Ala Val
          65             70             75             80
Asn Ile Ala Tyr Ala Cys Lys Leu Leu Glu Pro Asp Asp Lys Leu Phe
          85             90             95
Ile Leu Asn Thr Gln Ser Lys Asp Ala Cys Gly Met Leu Met Ser Thr
          100            105            110
Ile Leu Lys Glu Leu Gln Lys Lys Thr Gln Ala Leu Pro Glu Gln Val
          115            120            125
Ser Leu Ser Glu Asp Leu Leu Gln Pro Pro Val Pro Arg Asp Ser Gly
          130            135            140
Leu Arg Ala Gly Leu Ile Ile Thr Gly Lys Thr Leu Glu Phe Ala Leu
          145            150            155            160
Gln Glu Ser Leu Gln Lys Gln Phe Leu Glu Leu Thr Ser Trp Cys Gln
          165            170            175
Ala Val Val Cys Cys Arg Ala Thr Pro Leu Gln Lys Ser Glu Val Val
          180            185            190

```

-continued

Lys Leu Val Arg Ser His Leu Gln Val Met Thr Leu Ala Ile Gly Asp
 195 200 205
 Gly Ala Asn Asp Val Ser Met Ile Gln Val Ala Asp Ile Gly Ile Gly
 210 215 220
 Val Ser Gly Gln Glu Gly Met Gln Ala Val Met Ala Ser Asp Phe Ala
 225 230 235 240
 Val Ser Gln Phe Lys His Leu Ser Lys Leu Leu Leu Val His Gly His
 245 250 255
 Trp Cys Tyr Thr Arg Leu Ser Asn Met Ile Leu Tyr Phe Phe Tyr Lys
 260 265 270
 Asn Val Ala Tyr Val Asn Leu Leu Phe Trp Tyr Gln Phe Phe Cys Gly
 275 280 285
 Phe Ser Gly Thr Ser Met Thr Asp Tyr Trp Val Leu Ile Phe Phe Asn
 290 295 300
 Leu Leu Phe Thr Ser Ala Pro Pro Val Ile Tyr Gly Val Leu Glu Lys
 305 310 315 320
 Asp Val Ser Ala Glu Thr Leu Met Gln Leu Pro Glu Leu Tyr Arg Ser
 325 330 335
 Gly Gln Lys Ser Glu Ala Tyr Leu Pro His Thr Phe Trp Ile Thr Leu
 340 345 350
 Leu Asp Ala Phe Tyr Gln Ser Leu Val Cys Phe Phe Val Pro Tyr Phe
 355 360 365
 Thr Tyr Gln Gly Ser Asp Thr Asp Ile Phe Ala Phe Gly Asn Pro Leu
 370 375 380
 Asn Thr Ala Thr Leu Phe Ile Val Leu Leu His Leu Val Ile Glu Ser
 385 390 395 400
 Lys Ser Leu Thr Trp Ile His Leu Leu Val Ile Ile Gly Ser Ile Leu
 405 410 415
 Ser Tyr Phe Leu Phe Ala Ile Val Phe Gly Ala Met Cys Val Thr Cys
 420 425 430
 Asn Pro Pro Ser Asn Pro Tyr Trp Ile Met Gln Glu His Met Leu Asp
 435 440 445
 Pro Val Phe Tyr Leu Val Cys Ile Leu Thr Thr Ser Ile Ala Leu Leu
 450 455 460
 Pro Arg Phe Val Tyr Arg Val Leu Gln Gly Ser Leu Phe Pro Ser Pro
 465 470 475 480
 Ile Leu Arg Ala Lys His Phe Asp Arg Leu Thr Pro Glu Glu Arg Thr
 485 490 495
 Lys Ala Leu Lys Lys Trp Arg Gly Ala Gly Lys Met Asn Gln Val Thr
 500 505 510
 Ser Lys Tyr Ala Asn Gln Ser Ala Gly Lys Ser Gly Arg Arg Pro Met
 515 520 525
 Pro Gly Pro Ser Ala Val Phe Ala Met Lys Ser Ala Thr Ser Cys Ala
 530 535 540
 Ile Glu Gln Gly Asn Leu Ser Leu Cys Glu Thr Ala Leu Asp Gln Gly
 545 550 555 560
 Tyr Ser Glu Thr Lys Ala Phe Glu Met Ala Gly Pro Ser Lys Gly Lys
 565 570 575
 Glu Ser

<210> SEQ ID NO 17
 <211> LENGTH: 5958
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

-continued

<400> SEQUENCE: 17

```

gtcagctaca caacctggat cttaccacag tttggatatg actgaggctc tccaatgggc   60
cagatatcac tggcgacggc tgatcagagg tgcaaccagg gatgatgatt cagggccata   120
caactattcc tcgttgctcg cctgtgggcg caagtccctc cagatcccta aactgtcagg   180
aaggcaaccg attgttggtc cccacatcca gcccttcaag gatgagtagt agaagttctc   240
cggagcctat gtgaacaatc gaatacgaac aacaaagtac acacttctga attttgtgcc   300
aagaaattta tttgaacaat ttcacagagc tgccaattta tatttcctgt tcctagtgtg   360
cctgaactgg gtacctttgg tagaagcctt ccaaaaggaa atcacatgtg tgcctctggt   420
ggtggctcct acaattatcg caattaaaga tggcctggaa gattatcgga aatacaaaat   480
tgacaaacag atcaataatt taataactaa agtttatagt aggaagaga aaaaatacat   540
tgaccgatgc tggaaagacg ttactgttgg ggactttatt cgcctctcct gcaacgaggt   600
catccctgca gacatggtac tactcttttc cactgatcca gatggaatct gtcacattga   660
gacttctggt cttgatggag agagcaattt aaaacagagg cagggtggtc ggggatatgc   720
agaacaggac tctgaagtgt atcctgagaa gttttccagt aggatagaat gtgaagccc   780
aaacaatgac ctcagcagat tccgaggctt cctagaacat tccaacaaag aacgcgtggg   840
tctcagtaaa gaaaatttgt tgcttagagg atgcaccatt agaaacacag aggtctgtgt   900
gggcatttgt gtttatgcag gccatgaaac caaagcaatg ctgaacaaca gtgggccacg   960
gtataagcgc agcaaattag aaagaagagc aaacacagat gtcctctggt gtgtcatgct  1020
tctggtcata atgtgcttaa ctggcgaggt aggtcatgga atctggctga gcaggatga  1080
aaagatgcat tttttcaatg ttcccagacc tgatggacat atcatatcac cactgttggc  1140
aggattttat atgttttga ccatgatcat tttgttacag gtcttgatc ctatttctct  1200
ctatgtttcc atcgaaattg tgaagcttgg acaaatatat ttcattcaaa gtgatgtgga  1260
tttctacaat gaaaaaatgg attctattgt tcagtgccga gccctgaaca tcgccgagga  1320
tctgggacag attcagtacc tcttttccga taagacagga accctcactg agaataagat  1380
gggttttcca agatgtagtgt tggcaggatt tgattactgc catgaagaaa atgccaggag  1440
gttgagagtc tatcaggaag ctgtctctga agatgaagat tttatagaca cagtcagtgg  1500
ttccctcagc aatatggcaa aaccgagagc ccccagctgc aggacagtgc ataattgggc  1560
tttgggaaat aagccctcaa atcatcttgc tgggagctct tttactctag gaagtggaga  1620
aggagccagt gaagtgcctc attccagaca ggctgcttcc agtagcccca ttgaacaga  1680
cgtggtacca gacaccaggc ttttagacaa atttagtcag attacacctc ggctctttat  1740
gccactagat gagaccatcc aaaatccacc aatggaaact ttgtacatta tcgacttttt  1800
cattgcattg gcaatttgca acacagtagt ggtttctgct cctaaccaac cccgacaaaa  1860
gatcagacac ccttcaactg gggggttgcc cattaagtct ttggaagaga ttaaaagtct  1920
tttcagaga tggctctgtc gaagatcaag ttctccatcg cttacacagt ggaagagacc  1980
atcttctgga gttccaaacg cttttgtgag cagactccct ctctttagtc gaatgaaacc  2040
agcttcacct ttggagggaag aggtctccca ggtgtgtgag agccccaggt gctccagtag  2100
ctcagcttgc tgcacagaaa cagagaaaca acacggtagt gcaggcctcc tgaatggcaa  2160
ggcagagtcc ctccctggac agccattggc ctgcaacctg tgttatgagg ccgagagccc  2220
agacgaagcg gccttagtgt atgccgccag ggcttaccaa tgcactttac ggtctcggac  2280

```

-continued

accagagcag gtcattggtg actttgctgc ttggggacca ttaacatttc aactcctaca	2340
catcctgccc ttgactcag taagaaaaag aatgtctgtt gtggccgac accctctttc	2400
caatcaagtt gtggtgtata cgaaggcgc tgattctgtg atcatggagt tactgtcggg	2460
ggcttcccc gatggagcaa gtctggagaa acaacagatg atagtaaggg agaaaaacca	2520
gaagcacttt tttcttccat ttcaggtgtc gtgaaaagct tgaattcggc gcgccagata	2580
tcacgcgtgc caagggactg gctcaggatg actatgccaa acaaggcctt cgtactttat	2640
gtatagcaaa gaaggctcat agtgacactg aatatgcaga gtggctgagg aatcattttt	2700
tagctgaaac cagcattgac aacagggaag aattactact tgaatctgcc atgaggttg	2760
agaacaaact tacattactt ggtgctactg gcattgaaga ccgtctgcag gagggagtcc	2820
ctgaatctat agaagctctt cacaagcgg gcatacaagt ctggatgctg acaggggaca	2880
agcaggagac agctgtcaac atagcttatg catgcaaaact actggagcca gatgacaagc	2940
ttttatcct caatacccaa agtaaatgc gtatatgag attaaatctg ttcttctgta	3000
ttttcaaagg cattggaaca ttgagattt gatgtatgca aggattaaaa aaatgcctgt	3060
gggatgctga tgagcacaat ttgaaagaa cttcagaaga aaactcaagc cctgccagag	3120
caagtgtcat taagtgaaga ttacttcag cctcctgtcc cccgggactc aggggtacga	3180
gctggactca ttatcactg gaagaccctg gagtttgccc tgcaagaaag tctgcaaaag	3240
cagttcctgg aactgacatc ttggtgtcaa gctgtggtct gctgccgagc cacaccgctg	3300
cagaaaagtg aagtgtgaa attggtccgc agccatctcc aggtgatgac cctgtctatt	3360
ggtgagtga gatgaatctg agtcctgtc ttctcccttt cacaccacac cagacaccga	3420
tccttctgtc tctttcttct cccactgttc cttccatttt cctcctccct ttttctctac	3480
cacattcatg ccttcccac acctatttga gcaccttct ccatcaccta tttgagcacc	3540
ttctgtgaac caggtaatag ggtgtgaca tggtaacaa tacagtagtc cagacttctt	3600
agttcagtg cagaccccca aatcaacaag cttaaatcaa gtaataaact gaatcacaga	3660
actgaaaaat ccattgttgc taccttcagg aaagctaaat tcaaggacat gagaattcat	3720
ttctttatcc attccacaag tatttatcaa gtgccttttt tgtaccaggc atttttctag	3780
atggagatac aagagtatat aaaattggca aactaccttt ttacaaggaa cttacatcta	3840
gtaggaaagg atgcagtaa acaaagcata atctgtcagg ttcaggtagt gataagtact	3900
attgaaaaaa taagtggatg aggcacgta tagcactgga gatgggctgg ggctgctctt	3960
taaatcgatt tcaagagcta ctgtaagttg actgggagca gagatgtgaa ggaatcata	4020
aggggccatg gagacatggt ggtgccaatg atgttagcat gatacaagtg gcagacattg	4080
ggataggggt ctcaggtaaa gaaggcatgc aggcgtgat ggccagtga tttgccgttt	4140
ctcagttcaa acatctcagc aagctccttc ttgtccatgg aactgggtgt tatacacggc	4200
ttccaacat gattctctat tttttctata agaattgtgc ctatgtgaac ctcttttct	4260
ggtaccagtt cttttgtgga ttttcaggaa catccatgac tgattactgg gttttgatct	4320
tcttcaacct cctcttcaca tctgcccctc ctgtcattta tgggttttg gaaaaagatg	4380
tgtctgcaga gacctcatg caactgcctg aactttacag aagtggtcag aaatcagagg	4440
catacttacc ccataccttc tggatcacct tattggatgc tttttatcaa agcctggtct	4500
gcttctttgt gccttatttt acctaccagg gctcagatac tgacatcttt gcatttgaa	4560
acccctgaa cacagccact ctgttcacg ttctcctcca tctggtcatt gaaagcaaga	4620
gtttgaccag gtgcagtga tcacacctgc aattccagag ctttgggagg ctgtggatca	4680

-continued

```

catgaagcta agagttcaag accagcctgg gcaacataac ttggattcac ttgctggtca 4740
tcattggtag catcttgtct tattttttat ttgccatagt ttttgagcc atgtgtgtaa 4800
cttgcaacc accatccaac cttactgga ttatgcagga gcacatgctg gatccagtat 4860
tctacttagt ttgtatcctc acgacgtcca ttgctcttct gccagggttt gtatacagag 4920
ttcttcaggg atccctgttt ccattccaa ttctgagagc taagcacttt gacagactaa 4980
ctccagagga gaggactaaa gctctcaaga agtggagagg ggctggaaag atgaatcaag 5040
tgacatcaaa gtatgctaac caatcagctg gcaagtcagg aagaagaccc atgcttgccc 5100
cttctgctgt atttgcaatg aagtcagcaa cttcctgtgc tattgagcaa ggaaacttat 5160
ctctgtgtga aactgcttta gatcaaggct actctgaaac taaggccttt gagatggctg 5220
gacctccaa aggtaaagaa agctagatag cctccttgga gttgcaagta ttctttcaag 5280
gttggaagag ggattttgaa gaggtatctc tccaagcaag aatgacttgt ttttccataa 5340
gggacatgag cattttacta ggcttggaag agctgacatg atgagcatta ttgtatgttt 5400
gtatatacat ttgtgtaga gggctagagt ttgacctaga gagagtttaa ggaagtgaag 5460
tatttaattc agaaccaaat gcttttgtaa aactttttgg attttgtaaa agcattttca 5520
ttctcttaga aattcaagta ttttcaaggg gagtcatttg agatatattt attttactag 5580
gagatcttat attctagga aatgctttaa atggtcaggc tccaatcgga atttttttaa 5640
gaaaaaagta gtttttaata cattgggttag gactcagagg aaatacggaa aaaacattgt 5700
agatggtaat ttacagataa aatcccaaga gcctttaaac aacaaggtag ctaaataagg 5760
tataattata ctgcttaaaa tacaggtagt gcctattaat agctttttat ttcctatggg 5820
gagatgcttt ggtcttctgg ctgagatgta ggcatacctc tcaactcattt caatgctttc 5880
ctgagggtga gccttcattg gaaaggggaa agaggggtct aggttcacaa gggaccagga 5940
atgctttcct ctggcagg 5958

```

40

What is claimed is:

1. An isolated nucleic acid molecule comprising the novel human ATPase nucleotide sequence described in SEQ ID NO: 13.

2. An isolated nucleic acid molecule comprising a novel human ATPase nucleotide sequence that:

(a) encodes the amino acid sequence shown in SEQ ID NO:14; and

(b) hybridizes under highly stringent conditions to the nucleotide sequence of SEQ ID NO:13, wherein the

highly stringent conditions are: hybridization in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 65° C. and washing in 0.1×SSC/0.1% SDS at 68° C.

3. An isolated nucleic acid molecule comprising a human ATPase nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 14.

* * * * *

A handwritten checkmark symbol, consisting of a single continuous stroke that starts at the top left, curves down and to the right, and then loops back up and to the left to meet the starting point.

This Page Blank (uspto)



US006448388B1

(12) **United States Patent**
Friddle et al.(10) **Patent No.: US 6,448,388 B1**
(45) **Date of Patent: Sep. 10, 2002**(54) **HUMAN PROTEASES AND
POLYNUCLEOTIDES ENCODING THE
SAME**(75) **Inventors: Carl Johan Friddle, The Woodlands;
Erin Hilbun, Houston, both of TX
(US)**(73) **Assignee: Lexicon Genetics Incorporated, The
Woodlands, TX (US)**(*) **Notice:** Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.(21) **Appl. No.: 09/930,872**(22) **Filed: Aug. 15, 2001****Related U.S. Application Data**(60) **Provisional application No. 60/225,852, filed on Aug. 16,
2000.**(51) **Int. Cl.⁷ C12N 9/64; C12N 15/57**(52) **U.S. Cl. 536/23.2; 435/226; 435/219**(58) **Field of Search 435/219, 226;
536/23.2**(56) **References Cited****U.S. PATENT DOCUMENTS**

4,215,051 A	7/1980	Schroeder et al.	260/346
4,376,110 A	3/1983	David et al.	436/513
4,594,595 A	6/1986	Struckman	343/770
4,631,211 A	12/1986	Houghten	428/35
4,689,405 A	8/1987	Frank et al.	536/27
4,713,326 A	12/1987	Dattagupta et al.	435/6
4,873,191 A	10/1989	Wagner et al.	435/172.3
4,946,778 A	8/1990	Ladner et al.	435/69.6
5,252,743 A	10/1993	Barrett et al.	548/303.7
5,424,186 A	6/1995	Fodor et al.	435/6
5,445,934 A	8/1995	Fodor et al.	435/6
5,459,127 A	10/1995	Felgner et al.	514/7
5,556,752 A	9/1996	Lockhart et al.	435/6
5,700,637 A	12/1997	Southern	435/6
5,744,305 A	4/1998	Fodor et al.	435/6
5,830,721 A	11/1998	Stemmer et al.	435/172.1
5,837,458 A	11/1998	Minshull et al.	435/6
5,869,336 A	2/1999	Meyer et al.	435/348
5,877,397 A	3/1999	Lonberg et al.	800/2
5,948,767 A	9/1999	Scheule et al.	514/44
6,075,181 A	6/2000	Kucherlapati et al.	800/25
6,110,490 A	8/2000	Thierry	424/450
6,150,584 A	11/2000	Kucherlapati et al.	800/18

OTHER PUBLICATIONS

Hurskainen TL, Hirohata S, Seldin MF, Apte SS. (1999) ADAM-TS5, and ADAM T-S6, and ADAM-TS7, novel members of a new family of zinc metalloproteases. General features and genomic distribution of the ADAM-TS family. *J Biol Chem.*;274(36):25555-63.*
Sequence alignment SEQ ID No.: 4 with AF140675, ADAM-TS7.*
Bird et al, 1988, "Single-Chain Antigen-Binding Proteins", *Science* 242:423-426.

Bitter et al, 1987, "Expression and Secretion Vectors for Yeast", *Methods in Enzymology* 153:516-544.

Colbere-Garapin et al, 1981, "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells", *J. Mol. Biol.* 150:1-14.

Gautier et al, 1987 "α-DNA IV:α-anomeric and β-anomeric tetrathymidylates covalently linked to intercalating oxazolopyridocarbazole. Synthesis, physicochemical properties and poly (rA) binding", *Nucleic Acids Research* 15(16):6625-6641.

Gordon, 1989, "Transgenic Animals", *International Review of Cytology*, 115:171-229.

Greenspan et al, 1993, "Idiotypes: structure and immunogenicity", *FASEB Journal* 7:437-434.

Gu et al, 1994, "Deletion of DNA Polymerase β Gene Segment in T Cells Using Type-Specific Gene Targeting", *Science* 265:103-106.

Huse et al, 1989, "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", *Science* 246:1275-1281.

Huston et al, 1988, "Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*", *Proc. Natl. Acad. Sci. USA* 85:5879-5883.

Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and R Nase H", *FEBS Letters* 215(5):327-330.

Inoue et al, 1987, "Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides", *Nucleic Acids Research* 15(15):6131-6149.

Inouye & Inouye, 1985, "Up-promoter mutations in lpp gene of *Escherichia coli*", *Nucleic Acids Research* 13(9):3101-3110.

Janknecht et al, 1991, "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant Avaccinova virus", *PNAS* 88:8972-8976.

Kohler & Milstein, 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity", *Nature* 256:495-497.

Lasko et al, 1992, "Targeted oncogene activation by site-specific recombination in transgenic mice", *Proc. Natl. Acad. Sci. USA* 89:6232-6236.

Lavitrano et al, 1989, "Sperm Cells ad Vectors for Introducing Foreign DNA into Eggs: Genetic Transformation of Mice", *Cell* 57:717-723.

Lo, 1983, "Transformation by Iontophoretic Microinjection of DNA: Multiple Integrations without Tandem Insertions", *Mol. & Cell Biol* 3(10):1803-1814.

Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection", *Proc. Natl. Acad. Sci. USA* 81:3655-3659.

(List continued on next page.)

Primary Examiner—Rebecca E. Prouty
Assistant Examiner—Sheridan L Swope

(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are disclosed that can be used in therapeutic, diagnostic, and pharmacogenomic applications.

5 Claims, No Drawings

OTHER PUBLICATIONS

- Lowy et al, 1980, "Isolation of Transforming DNA: cloning the Hamster apt Gene", Cell 22:817-823.
- Morrison et al, 1984, "Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains", Proc. Natl. Acad. Sci. USA 81:6851-6855.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", Proc. Natl. Acad. Sci. USA 78(4):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies processing novel effector functions", Nature 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", J. of Immunology 147:2429-2438.
- O'Hare et al, 1981, "Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase", Proc. Natl. Acad. Sci. USA 78(3):1527-1531.
- Ruther et al, 1983, "Easy identification of cDNA clones", EMBO Journal 2(10):1791-1794.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", Gene 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", Proc. Natl. Acad. Sci. USA 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", J. Virol. 46(2):584-593.
- Stein et al, 1988, "Physiochemical properties of phosphorothioate oligodeoxynucleotides", Nucleic Acid Research 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", Proc. Natl. Acad. Sci. USA 48:2026-2034.
- Takeda et al, 1985, "Construction of Chimaeri processed immunoglobulin genes containing mouse variable and human constant region sequences", Nature 314:452-454.
- Thompson et al, 1989, "Germ Line Transmission and Expression of a Corrected HPRT Gene Produced by Gene Targeting in Embryonic Stem Cells", Cell 56:313-321.
- Van Der Putten et al, 1985, "Efficient insertion of genes into the mouse germ line via retroviral vectors", Proc. Natl. Acad. Sci. USA 82:6148-6152.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", J. Biol. Chemistry 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase to Cultured Mouse Cells", 11:223-232.
- Wigler et al, 1980, "Transformation mammalian cells with an amplifiable dominant-acting gene", Proc. Natl. Acad. Sci. USA 77(6):3567-3570.

* cited by examiner

1

HUMAN PROTEASES AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application Number 60/225,852 which was filed on Aug. 16, 2000 and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins sharing sequence similarity with mammalian proteases. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed sequences, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed polynucleotides that can be used for diagnosis, drug screening, clinical trial monitoring, or the treatment of physiological disorders or diseases.

2. BACKGROUND OF THE INVENTION

Proteases cleave protein substrates as part of degradation, maturation, and secretory pathways within the body. Proteases have been associated with, inter alia, regulating development, diabetes, obesity, infertility, modulating cellular processes, and infectious disease.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins, and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal proteases and particularly zinc metalloproteases.

The novel human nucleic acid (cDNA) sequences described herein, encode proteins/open reading frames (ORFs) of 491 and 1224 amino acids in length (see SEQ ID NOS: 2 and 4 respectively).

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof that compete with native NHPs, NHP peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHPs (e.g., expression constructs that place the described gene under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" (which can be conditional) that do not express a functional NHP.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP products, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

2

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequences of several NHP ORFs encoding the described NHP amino acid sequences. SEQ ID NO:5 describes a NHP ORF and flanking sequences.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPs described for the first time herein are novel proteins that are expressed in, inter alia, human cell lines, and human fetal brain, brain, pituitary, cerebellum, spinal cord, thymus, lymph node, trachea, kidney, fetal liver, prostate, testis, thyroid, adrenal gland, pancreas, small intestine, colon, skeletal muscle, heart, uterus, mammary gland, adipose, esophagus, bladder, cervix, pericardium, ovary, fetal kidney, and fetal lung cells.

The described sequences were compiled from cDNA clones, genomic sequence, and cDNAs derived from human kidney, mammary gland, and cerebellum mRNAs (Edge Biosystems, Gaithersburg, Md., and Clontech, Palo Alto, Calif.). The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHPs, and NHP products; (b) nucleotides that encode one or more portions of a NHP that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of a NHP, or one of its domains (e.g., a receptor or ligand binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF), or a contiguous exon splice junction first described in the Sequence Listing, that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, supra), yet still encode

a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding a NHP ORF, or its functional equivalent, encoded by a polynucleotide sequence that is about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP gene nucleotide sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length may partially overlap each other and/or a NHP sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described NHP polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 18, and preferably about 25, nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences may begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to

5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof. In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide

polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from human or nonhuman cell lines or tissue known or suspected to express an allele of a NHP gene. The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene, such as, for example, testis tissue). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, connective tissue disorders, infertility, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the

corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue can be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, AP-NHP or NHP-AP fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869, 336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation) or genetically engineered transcription factor. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus hCMV immediate early gene, regulatable, viral (particularly retroviral LTR promoters) the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The

NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor for a NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHP, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics a NHP could activate or effectively antagonize the endogenous NHP receptor. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products in vivo; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHP, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 THE NHP SEQUENCES

The cDNA sequences and corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing. SEQ ID NO:5 describes a NHP ORF as well as flanking regions. The NHP nucleotides were obtained from human cDNA libraries using probes and/or primers generated from human genomic sequence. Expression analysis has provided evidence that the described NHP can be expressed a variety of human cells.

5.2 NHPs AND NHP POLYPEPTIDES

The NHPs, NHP polypeptides, NHP peptide fragments, mutated, truncated, or deleted forms of NHP, and/or NHP fusion proteins can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to a NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease. The described NHPs share similarity with a variety of proteases, including proteases having thrombospondin repeats, disintegrins, aggrecanases, and metalloproteinases (especially zinc metalloproteases of the ADAMTS family).

The Sequence Listing discloses the amino acid sequences encoded by the described NHP polynucleotides. The NHPs display an initiator methionines in DNA sequence contexts

consistent with a translation initiation site, and several of the ORFs display a signal-like sequence which can indicate that the described NHP ORFs are secreted proteins or can be membrane associated.

The NHP amino acid sequences of the invention include the amino acid sequences presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHPs encoded by a NHP nucleotide sequence described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biolog", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a substrate of a NHP, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where, as in the present instance, a NHP peptide or NHP polypeptide is thought to be a soluble or secreted molecule, the peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express NHP, or functional equivalent, in situ. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of a NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms

such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP encoding nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5 K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of and/or containing a NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Pharmacia or American Type Culture Collection) can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polydrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP gene coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a

NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express a NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of a NHP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthineguanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp⁺ or apr⁺ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci.

USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

5.3 ANTIBODIES TO NHP PRODUCTS

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab)₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of a NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with a NHP, an NHP peptide (e.g., one corresponding to a functional domain of a NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of a NHP or mutated variants of a NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by

any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc.

Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 341:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:127-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J. 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor can be used to generate anti-idiotypes that "mimic" a NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP signaling pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 5

<210> SEQ ID NO 1

<211> LENGTH: 1476

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atgaagcccc gcgcgcgcgg atggcggggc ttggcggcgc tgtggatgct gttggcgcag    60
gtggccgagc aggcacatgc gtgcgccatg ggaccgcgag cggcagcgcc tgggagcccg    120
agcgtccccgc gtctctctcc acccgcggag cggccgggct ggatggaaaa gggcgaatat    180
gacctgggtct ctgcctacga ggttgaccac aggggcgatt acgtgtccca tgaatcatg    240
caccatcagc ggcggagaag agcagtggcc gtgtccgagg ttgagtctct tcaccttcgg    300
ctgaaaggct ccaggcacga cttccacgtg gatctgagga cttccagcag cctagtggct    360
cctggcttta ttgtgcagac gttgggaaag acaggcacta agtctgtgca gactttaccg    420
ccagaggact tctgtttcta tcaaggctct ttgcgatcac acagaaactc ctcatgtggc    480
ctttcaacct gccaaaggct gtccagcatg atacgaacag aagaggcaga ttacttccta    540
aggccacttc cttcacacct ctcatggaaa ctgcgcagag ctgcccaagg cagctcgcca    600
tcccacgtac tgtacaagag atccacagag ccccatgctc ctggggccag tgaggctctg    660
gtgacctcaa ggacatggga gctggcacat caaccctgc acagcagcga ccttcgcctg    720
ggactgccac aaaagcagca tttctgtgga agacgcaaga aatacatgcc ccagcctccc    780
aaggaagacc tcttcatctt gccagatgag tataagtctt gcttacggca taagcgctct    840
cttctgaggt cccatagaaa tgaagaactg aacgtggaga ccttgggtgt ggtcgacaaa    900
aagatgatgc aaaccatgg ccatgaaaat atcaccacct acgtgctcac gataactaac    960
atggtatctg ctttatitcaa agatggaaca ataggaggaa acatcaacat tgcaattgta   1020
ggctctgattc ttctagaaga tgaacagcca ggactgggtg taagtcacca cgcagaccac   1080
accttaagta gcttctgcca gtggcagtct ggattgatgg ggaaagatgg gactcgtcat   1140
gaccacgcca tcttactgac tggcttggtg atatgttctt ggaagaatga gccctgtgac   1200
actttgggat ttgcacccat aagtggaaat tgtagtaaat atcgagctg cagattaat   1260
gaagatacag gtcttggact ggccttcacc attgccatg agtctggaca caactttggc   1320
atgattcatg atggagaagg gaacatgtgt aaaaagtccg agggcaacat catgtcccct   1380
acattggcag gacgcaatgg agtcttctcc tggtcacct gcagccgcca gtatctacac   1440
aaatttctaa gatcagtga aatgccagct ctctga                               1476

```

<210> SEQ ID NO 2

<211> LENGTH: 491

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

```

Met Lys Pro Arg Ala Arg Gly Trp Arg Gly Leu Ala Ala Leu Trp Met
 1             5             10             15

Leu Leu Ala Gln Val Ala Glu Gln Ala Pro Ala Cys Ala Met Gly Pro
 20             25             30

Ala Ala Ala Ala Pro Gly Ser Pro Ser Val Pro Arg Pro Pro Pro Pro
 35             40             45

```


-continued

Ala	Glu	Arg	Pro	Gly	Trp	Met	Glu	Lys	Gly	Glu	Tyr	Asp	Leu	Val	Ser	
50						55					60					
Ala	Tyr	Glu	Val	Asp	His	Arg	Gly	Asp	Tyr	Val	Ser	His	Glu	Ile	Met	
65					70					75				80		
His	His	Gln	Arg	Arg	Arg	Arg	Ala	Val	Ala	Val	Ser	Glu	Val	Glu	Ser	
				85						90				95		
Leu	His	Leu	Arg	Leu	Lys	Gly	Ser	Arg	His	Asp	Phe	His	Val	Asp	Leu	
				100						105				110		
Arg	Thr	Ser	Ser	Ser	Leu	Val	Ala	Pro	Gly	Phe	Ile	Val	Gln	Thr	Leu	
		115						120				125				
Gly	Lys	Thr	Gly	Thr	Lys	Ser	Val	Gln	Thr	Leu	Pro	Pro	Glu	Asp	Phe	
	130					135						140				
Cys	Phe	Tyr	Gln	Gly	Ser	Leu	Arg	Ser	His	Arg	Asn	Ser	Ser	Val	Ala	
145					150					155				160		
Leu	Ser	Thr	Cys	Gln	Gly	Leu	Ser	Gly	Met	Ile	Arg	Thr	Glu	Glu	Ala	
				165					170					175		
Asp	Tyr	Phe	Leu	Arg	Pro	Leu	Pro	Ser	His	Leu	Ser	Trp	Lys	Leu	Gly	
			180						185					190		
Arg	Ala	Ala	Gln	Gly	Ser	Ser	Pro	Ser	His	Val	Leu	Tyr	Lys	Arg	Ser	
	195						200						205			
Thr	Glu	Pro	His	Ala	Pro	Gly	Ala	Ser	Glu	Val	Leu	Val	Thr	Ser	Arg	
	210					215						220				
Thr	Trp	Glu	Leu	Ala	His	Gln	Pro	Leu	His	Ser	Ser	Asp	Leu	Arg	Leu	
225					230					235				240		
Gly	Leu	Pro	Gln	Lys	Gln	His	Phe	Cys	Gly	Arg	Arg	Lys	Lys	Tyr	Met	
			245						250					255		
Pro	Gln	Pro	Pro	Lys	Glu	Asp	Leu	Phe	Ile	Leu	Pro	Asp	Glu	Tyr	Lys	
			260					265					270			
Ser	Cys	Leu	Arg	His	Lys	Arg	Ser	Leu	Leu	Arg	Ser	His	Arg	Asn	Glu	
		275					280						285			
Glu	Leu	Asn	Val	Glu	Thr	Leu	Val	Val	Val	Asp	Lys	Lys	Met	Met	Gln	
	290					295					300					
Asn	His	Gly	His	Glu	Asn	Ile	Thr	Thr	Tyr	Val	Leu	Thr	Ile	Leu	Asn	
305					310					315				320		
Met	Val	Ser	Ala	Leu	Phe	Lys	Asp	Gly	Thr	Ile	Gly	Gly	Asn	Ile	Asn	
			325						330					335		
Ile	Ala	Ile	Val	Gly	Leu	Ile	Leu	Leu	Glu	Asp	Glu	Gln	Pro	Gly	Leu	
			340					345					350			
Val	Ile	Ser	His	His	Ala	Asp	His	Thr	Leu	Ser	Ser	Phe	Cys	Gln	Trp	
	355						360						365			
Gln	Ser	Gly	Leu	Met	Gly	Lys	Asp	Gly	Thr	Arg	His	Asp	His	Ala	Ile	
	370					375					380					
Leu	Leu	Thr	Gly	Leu	Asp	Ile	Cys	Ser	Trp	Lys	Asn	Glu	Pro	Cys	Asp	
385				390						395				400		
Thr	Leu	Gly	Phe	Ala	Pro	Ile	Ser	Gly	Met	Cys	Ser	Lys	Tyr	Arg	Ser	
			405						410					415		
Cys	Thr	Ile	Asn	Glu	Asp	Thr	Gly	Leu	Gly	Leu	Ala	Phe	Thr	Ile	Ala	
		420						425					430			
His	Glu	Ser	Gly	His	Asn	Phe	Gly	Met	Ile	His	Asp	Gly	Glu	Gly	Asn	
	435						440					445				
Met	Cys	Lys	Lys	Ser	Glu	Gly	Asn	Ile	Met	Ser	Pro	Thr	Leu	Ala	Gly	
	450					455					460					
Arg	Asn	Gly	Val	Phe	Ser	Trp	Ser	Pro	Cys	Ser	Arg	Gln	Tyr	Leu	His	

-continued

465	470	475	480	
Lys Phe Leu Arg Ser Val Lys Met Pro Ala Leu				
	485	490		

<210> SEQ ID NO 3
 <211> LENGTH: 3675
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens
 <400> SEQUENCE: 3

atgaagcccc	gcgcgcgcgc	atggcggggc	ttggcggcgc	tgtggatgct	gctggcgcag	60
gtggccgagc	aggcacctgc	gtgcgccatg	ggacccgcag	cggcagcgcc	tgggagcccg	120
agcgtccccg	gtcctcctcc	acccgcggag	cggccgggct	ggatggaaaa	ggcgaatat	180
gacctggtct	ctgcctacga	ggttgaccac	aggggcgatt	acgtgtccca	tgaatcatg	240
caccatcagc	ggcgagaag	agcagtggcc	gtgtccgagg	ttgagtctct	tcaccttcgg	300
ctgaaaggct	ccaggcacga	cttcacgtg	gatctgagga	cttcacagag	cctagtggct	360
cctggcttta	ttgtgcagac	gttgggaaa	acaggcacta	agtctgtgca	gactttaccg	420
ccagaggact	tctgtttcta	tcaaggctct	ttgcgatcac	acagaaactc	ctcagtggcc	480
ctttcaacct	gccaaaggct	gtcaggcatg	atacgaacag	aagaggcaga	ttacttccta	540
aggccacttc	cttcacacct	ctcatggaaa	ctcggcagag	ctgcccagg	cagctcgcca	600
tcccacgtac	tgtacaagag	atccacagag	ccccatgctc	ctggggccag	tgaggctctg	660
gtgacctcaa	ggacatggga	gctggcacat	caaccctgc	acagcagcga	ccttcgcctg	720
ggactgccac	aaaagcagca	tttctgtgga	agacgcaaga	aatacatgcc	ccagcctccc	780
aaggaagacc	tcttcatctt	gccagatgag	tataagtctt	gcttacggca	taagcgtctt	840
cttctgaggt	cccatagaaa	tgaagaactg	aacgtggaga	ccttggtggt	ggtcgacaaa	900
aagatgatgc	aaaaccatgg	ccatgaaaat	atcaccacct	acgtgctcac	gatactcaac	960
atggatatctg	ctttattcaa	agatggaaaca	ataggaggaa	acatcaacat	tgcaattgta	1020
ggtctgatcc	ttctagaaga	tgaacagcca	ggactggtga	taagtcacca	cgcagaccac	1080
accttaagta	gcttctgcca	gtggcagtct	ggattgatgg	ggaaagatgg	gactcgtcat	1140
gaccacgcca	tcttactgac	tggtctggat	atatgttcct	ggaagaatga	gccctgtgac	1200
actttgggat	ttgcacccat	aagtggaaatg	tgtagttaaat	atcgacagctg	cacgattaat	1260
gaagatacag	gtcttggaact	ggccttcacc	attgcccattg	agtctggaca	caactttggc	1320
atgattcatg	atggagaagg	gaacatgtgt	aaaaagtcgg	agggcaacat	catgtcccct	1380
acattggcag	gacgcaatgg	agtcttctcc	tggtcaccct	gcagccgcca	gtatctacac	1440
aaattttctaa	gcaccgctca	agctatctgc	cttgctgatac	agccaaagcc	tgtgaaggaa	1500
tacaagtatc	ctgagaaatt	gccaggagaa	ttatatgatg	caaacacaca	gtgcaagtgg	1560
cagttcggag	agaaagccaa	gctctgcatg	ctggacttta	aaaaggacat	ctgtaagccc	1620
ctgtggtgcc	atcgtattgg	aaggaaatgt	gagactaaat	ttatgccagc	agcagaaggc	1680
acaattttgtg	ggcatgacat	gtggtgccgg	ggaggacagt	gtgtgaaata	tggtgatgaa	1740
ggccccaagc	ccaccatagg	ccactggctg	gactggtctt	cttggtcccc	atgtccagg	1800
acctgcggag	ggggagtatc	tcataggagt	cgcctctgca	ccaaccccaa	gccatcgcat	1860
ggagggaagt	tctgtgaggg	ctccactcgc	actctgaagc	tctgcaacag	tcagaaatgt	1920
ccccgggaca	gtgttgactt	ccgtgctgct	cagtgtgccg	agcacaaacg	cagacgattc	1980

-continued

```

agagggcgcc actacaagtg gaagccttac actcaagtag aagatcagga cttatgcaaa 2040
ctctactgta tcgcagaagg atttgatttc ttottttctt tgcataataa agtcaaagat 2100
gggactccat gctcggagga tagccgtaat gtttgtagat atgggatatg tgagagagtt 2160
ggatgtgaca atgtccttgg atctgatgct gttgaagacg tctgtggggg gtgtaacggg 2220
aataactcag cctgcacgat tcacaggggt ctctacacca agcaccacca caccaaccag 2280
tattatcaca tggtcaccat tccttctgga gcccgagta tccgcatacta tgaaatgaac 2340
gtctctacct cctacatttc tgtgcgcaat gccctcagaa ggtactacct gaatgggcac 2400
tggaccgtgg actggccggc cgggtacaaa ttttcgggca ctactttcga ctacagacgg 2460
tcctataatg agcccgagaa cttaatcgct actggaccaa ccaacgagac actgattgtg 2520
gagctgctgt ttcagggaag gaacccgggt gttgcctggg aatactccat gcctcgcttg 2580
gggaccgaga agcagccccc tgcccagccc agctacactt gggccatcgt gcgctctgag 2640
tgctcctgtt cctgcggagg gggacagatg accgtgagag agggctgcta cagagacctg 2700
aagtttcaag taaatatgtc cttctgcaat cccaagacac gacctgtcac ggggctggtg 2760
ccttgcaaaq tatctgcctg tcctcccagc tggtcctggg ggaactggag tgcctgcagt 2820
cggacgtgtg gcgggggtgc ccagagccgc cccgtgcagt gcacacggcg ggtgcactat 2880
gactcggagc cagtcccgcc cagcctgtgc cctcagcctg ctccctccag caggcaggcc 2940
tgcaactctc agagctgccc acctgcatgg agcgcggggc cctgggcaga gtgctcacac 3000
acctgtggga aggggtggag gaagcgggca gtggcctgta agagcaccaa cccctcggcc 3060
agagcgcagc tgcgtcccga cgctgtctgc acctccgagc ccaagcccag gatgcatgaa 3120
gcctgtctgc ttcagcgtgc ccacaagccc aagaagctgc agtggctggt gtccgcctgg 3180
tcccagtgct ctgtgacatg tgaagagga acacagaaaa gattcttaaa atgtgctgaa 3240
aagtatgttt ctggaagta tcgagagctg gcctcaaga agtgctcaca ttgcccgaag 3300
cccagcctgg agctggaacg tgcttgccc ccgcttccat gcccagga cccccattt 3360
gctgtgctgg gaccctcag gggcagctgg tttgctcac cctgggtctc gtgcacggcc 3420
agctgtgggg gaggcgttca gacgaggtcc gtgcagtgcc tggctggggg ccggccggcc 3480
tcagggtgcc tcctgcacca gaagccttcg gcctccctgg cctgcaacac tcacttctgc 3540
cccattgcag agaagaaaga tgcttctgc aaagactact tccactggtg ctacctggtg 3600
ccccagcagc ggatgtgcag ccacaagttc tacggcaagc agtgctgcaa gacttgctct 3660
aagtccaact tgtga 3675

```

<210> SEQ ID NO 4

<211> LENGTH: 1224

<212> TYPE: PRT

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 4

```

Met Lys Pro Arg Ala Arg Gly Trp Arg Gly Leu Ala Ala Leu Trp Met
 1           5           10          15

```

```

Leu Leu Ala Gln Val Ala Glu Gln Ala Pro Ala Cys Ala Met Gly Pro
 20          25          30

```

```

Ala Ala Ala Ala Pro Gly Ser Pro Ser Val Pro Arg Pro Pro Pro Pro
 35          40          45

```

```

Ala Glu Arg Pro Gly Trp Met Glu Lys Gly Glu Tyr Asp Leu Val Ser
 50          55          60

```

```

Ala Tyr Glu Val Asp His Arg Gly Asp Tyr Val Ser His Glu Ile Met

```

-continued

65	70	75	80
His His Gln Arg Arg Arg Arg Ala Val Ala Val Ser Glu Val Glu Ser	85	90	95
Leu His Leu Arg Leu Lys Gly Ser Arg His Asp Phe His Val Asp Leu	100	105	110
Arg Thr Ser Ser Ser Leu Val Ala Pro Gly Phe Ile Val Gln Thr Leu	115	120	125
Gly Lys Thr Gly Thr Lys Ser Val Gln Thr Leu Pro Pro Glu Asp Phe	130	135	140
Cys Phe Tyr Gln Gly Ser Leu Arg Ser His Arg Asn Ser Ser Val Ala	145	150	155
Leu Ser Thr Cys Gln Gly Leu Ser Gly Met Ile Arg Thr Glu Glu Ala	165	170	175
Asp Tyr Phe Leu Arg Pro Leu Pro Ser His Leu Ser Trp Lys Leu Gly	180	185	190
Arg Ala Ala Gln Gly Ser Ser Pro Ser His Val Leu Tyr Lys Arg Ser	195	200	205
Thr Glu Pro His Ala Pro Gly Ala Ser Glu Val Leu Val Thr Ser Arg	210	215	220
Thr Trp Glu Leu Ala His Gln Pro Leu His Ser Ser Asp Leu Arg Leu	225	230	235
Gly Leu Pro Gln Lys Gln His Phe Cys Gly Arg Arg Lys Lys Tyr Met	245	250	255
Pro Gln Pro Pro Lys Glu Asp Leu Phe Ile Leu Pro Asp Glu Tyr Lys	260	265	270
Ser Cys Leu Arg His Lys Arg Ser Leu Leu Arg Ser His Arg Asn Glu	275	280	285
Glu Leu Asn Val Glu Thr Leu Val Val Val Asp Lys Lys Met Met Gln	290	295	300
Asn His Gly His Glu Asn Ile Thr Thr Tyr Val Leu Thr Ile Leu Asn	305	310	315
Met Val Ser Ala Leu Phe Lys Asp Gly Thr Ile Gly Gly Asn Ile Asn	325	330	335
Ile Ala Ile Val Gly Leu Ile Leu Leu Glu Asp Glu Gln Pro Gly Leu	340	345	350
Val Ile Ser His His Ala Asp His Thr Leu Ser Ser Phe Cys Gln Trp	355	360	365
Gln Ser Gly Leu Met Gly Lys Asp Gly Thr Arg His Asp His Ala Ile	370	375	380
Leu Leu Thr Gly Leu Asp Ile Cys Ser Trp Lys Asn Glu Pro Cys Asp	385	390	395
Thr Leu Gly Phe Ala Pro Ile Ser Gly Met Cys Ser Lys Tyr Arg Ser	405	410	415
Cys Thr Ile Asn Glu Asp Thr Gly Leu Gly Leu Ala Phe Thr Ile Ala	420	425	430
His Glu Ser Gly His Asn Phe Gly Met Ile His Asp Gly Glu Gly Asn	435	440	445
Met Cys Lys Lys Ser Glu Gly Asn Ile Met Ser Pro Thr Leu Ala Gly	450	455	460
Arg Asn Gly Val Phe Ser Trp Ser Pro Cys Ser Arg Gln Tyr Leu His	465	470	475
Lys Phe Leu Ser Thr Ala Gln Ala Ile Cys Leu Ala Asp Gln Pro Lys	485	490	495

-continued

Pro Val Lys Glu Tyr Lys Tyr Pro Glu Lys Leu Pro Gly Glu Leu Tyr
 500 505 510
 Asp Ala Asn Thr Gln Cys Lys Trp Gln Phe Gly Glu Lys Ala Lys Leu
 515 520 525
 Cys Met Leu Asp Phe Lys Lys Asp Ile Cys Lys Ala Leu Trp Cys His
 530 535 540
 Arg Ile Gly Arg Lys Cys Glu Thr Lys Phe Met Pro Ala Ala Glu Gly
 545 550 555 560
 Thr Ile Cys Gly His Asp Met Trp Cys Arg Gly Gly Gln Cys Val Lys
 565 570 575
 Tyr Gly Asp Glu Gly Pro Lys Pro Thr His Gly His Trp Ser Asp Trp
 580 585 590
 Ser Ser Trp Ser Pro Cys Ser Arg Thr Cys Gly Gly Gly Val Ser His
 595 600 605
 Arg Ser Arg Leu Cys Thr Asn Pro Lys Pro Ser His Gly Gly Lys Phe
 610 615 620
 Cys Glu Gly Ser Thr Arg Thr Leu Lys Leu Cys Asn Ser Gln Lys Cys
 625 630 635 640
 Pro Arg Asp Ser Val Asp Phe Arg Ala Ala Gln Cys Ala Glu His Asn
 645 650 655
 Ser Arg Arg Phe Arg Gly Arg His Tyr Lys Trp Lys Pro Tyr Thr Gln
 660 665 670
 Val Glu Asp Gln Asp Leu Cys Lys Leu Tyr Cys Ile Ala Glu Gly Phe
 675 680 685
 Asp Phe Phe Ser Leu Ser Asn Lys Val Lys Asp Gly Thr Pro Cys
 690 695 700
 Ser Glu Asp Ser Arg Asn Val Cys Ile Asp Gly Ile Cys Glu Arg Val
 705 710 715 720
 Gly Cys Asp Asn Val Leu Gly Ser Asp Ala Val Glu Asp Val Cys Gly
 725 730 735
 Val Cys Asn Gly Asn Asn Ser Ala Cys Thr Ile His Arg Gly Leu Tyr
 740 745 750
 Thr Lys His His His Thr Asn Gln Tyr Tyr His Met Val Thr Ile Pro
 755 760 765
 Ser Gly Ala Arg Ser Ile Arg Ile Tyr Glu Met Asn Val Ser Thr Ser
 770 775 780
 Tyr Ile Ser Val Arg Asn Ala Leu Arg Arg Tyr Tyr Leu Asn Gly His
 785 790 795 800
 Trp Thr Val Asp Trp Pro Gly Arg Tyr Lys Phe Ser Gly Thr Thr Phe
 805 810 815
 Asp Tyr Arg Arg Ser Tyr Asn Glu Pro Glu Asn Leu Ile Ala Thr Gly
 820 825 830
 Pro Thr Asn Glu Thr Leu Ile Val Glu Leu Leu Phe Gln Gly Arg Asn
 835 840 845
 Pro Gly Val Ala Trp Glu Tyr Ser Met Pro Arg Leu Gly Thr Glu Lys
 850 855 860
 Gln Pro Pro Ala Gln Pro Ser Tyr Thr Trp Ala Ile Val Arg Ser Glu
 865 870 875 880
 Cys Ser Val Ser Cys Gly Gly Gly Gln Met Thr Val Arg Glu Gly Cys
 885 890 895
 Tyr Arg Asp Leu Lys Phe Gln Val Asn Met Ser Phe Cys Asn Pro Lys
 900 905 910

-continued

Thr Arg Pro Val Thr Gly Leu Val Pro Cys Lys Val Ser Ala Cys Pro
 915 920 925
 Pro Ser Trp Ser Val Gly Asn Trp Ser Ala Cys Ser Arg Thr Cys Gly
 930 935 940
 Gly Gly Ala Gln Ser Arg Pro Val Gln Cys Thr Arg Arg Val His Tyr
 945 950 955 960
 Asp Ser Glu Pro Val Pro Ala Ser Leu Cys Pro Gln Pro Ala Pro Ser
 965 970 975
 Ser Arg Gln Ala Cys Asn Ser Gln Ser Cys Pro Pro Ala Trp Ser Ala
 980 985 990
 Gly Pro Trp Ala Glu Cys Ser His Thr Cys Gly Lys Gly Trp Arg Lys
 995 1000 1005
 Arg Ala Val Ala Cys Lys Ser Thr Asn Pro Ser Ala Arg Ala Gln Leu
 1010 1015 1020
 Leu Pro Asp Ala Val Cys Thr Ser Glu Pro Lys Pro Arg Met His Glu
 1025 1030 1035 1040
 Ala Cys Leu Leu Gln Arg Cys His Lys Pro Lys Lys Leu Gln Trp Leu
 1045 1050 1055
 Val Ser Ala Trp Ser Gln Cys Ser Val Thr Cys Glu Arg Gly Thr Gln
 1060 1065 1070
 Lys Arg Phe Leu Lys Cys Ala Glu Lys Tyr Val Ser Gly Lys Tyr Arg
 1075 1080 1085
 Glu Leu Ala Ser Lys Lys Cys Ser His Leu Pro Lys Pro Ser Leu Glu
 1090 1095 1100
 Leu Glu Arg Ala Cys Ala Pro Leu Pro Cys Pro Arg His Pro Pro Phe
 1105 1110 1115 1120
 Ala Ala Ala Gly Pro Ser Arg Gly Ser Trp Phe Ala Ser Pro Trp Ser
 1125 1130 1135
 Gln Cys Thr Ala Ser Cys Gly Gly Gly Val Gln Thr Arg Ser Val Gln
 1140 1145 1150
 Cys Leu Ala Gly Gly Arg Pro Ala Ser Gly Cys Leu Leu His Gln Lys
 1155 1160 1165
 Pro Ser Ala Ser Leu Ala Cys Asn Thr His Phe Cys Pro Ile Ala Glu
 1170 1175 1180
 Lys Lys Asp Ala Phe Cys Lys Asp Tyr Phe His Trp Cys Tyr Leu Val
 1185 1190 1195 1200
 Pro Gln His Gly Met Cys Ser His Lys Phe Tyr Gly Lys Gln Cys Cys
 1205 1210 1215
 Lys Thr Cys Ser Lys Ser Asn Leu
 1220

<210> SEQ ID NO 5

<211> LENGTH: 4042

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 5

```

ccttcccgcg ctctgcttgg gtcgggtcct ccctgccgcg tcgcacgctg ccggccgggg      60
accctccggt ggccctagc ccctcggagc gctcctggat gaagccccgc gcgcgcggat      120
ggcggggctt ggcggcgctg tggatgctgc tggcgaggt ggccgagcag gcacctgcgt      180
gcgccatggg accgcagcgc gcagcgcctg ggagccccgag cgtcccgcgt cctcctccac      240
ccgcgggagc gccgggctgg atggaaaagg gcgaatatga cctggtctct gcctacgagg      300
ttgaccacag gggcgattac gtgtcccatg aaatcatgca ccatcagcgg cggagaagag      360

```

-continued

cagtggccgt gtcgaggtt gagtctcttc accttcggct gaaaggctcc aggcacgact	420
tccacgtgga tctgaggact tccagcagcc tagtggctcc tggctttatt gtgcagacgt	480
tgggaaagac aggcactaag tctgtgcaga ctttaccgcc agaggacttc tgtttctatc	540
aaggctcttt gcgatcacac agaaactcct cagtggccct ttcaacctgc caaggcttgt	600
caggcatgat acgaacagaa gaggcagatt acttcctaag gccacttcct tcacacctct	660
catggaaact cggcagagct gccaaggca gctcggcctc ccacgtactg tacaagagat	720
ccacagagcc ccatgctcct ggggccagtg aggtcctggt gacctcaagg acatgggagc	780
tggcacatca acccctgcac agcagcgacc ttgcctggg actgccacaa aagcagcatt	840
tctgtggaag acgcaagaaa tacatgcccc agcctcccaa ggaagacctc ttcatcttgc	900
cagatgagta taagtcttgc ttacggcata agcgtctctt tctgaggctc catagaaatg	960
aagaactgaa cgtggagacc ttggtggtg tcgacaaaa gatgatgcaa aacctaggcc	1020
atgaaaatat caccacctac gtgctcacga tactcaacat ggtatctgct ttattcaaag	1080
atggaacaat agggagaaac atcaacattg caattgtagg tctgattctt ctagaagatg	1140
aacagccagg actggtgata agtcaccacg cagaccacac cttaagtagc ttctgccagt	1200
ggcagctctg attgatggg aaagatggga ctcgatcatga ccacgccatc ttactgactg	1260
gtctggatat atgttctctg aagaatgagc cctgtgacac ttggggattt gcaccataa	1320
gtggaatgtg tagtaaatat cgcagctgca cgattaatga agatacaggt cttggactgg	1380
ccttcaccat tgcccatgag tctggacaca actttggcat gattcatgat ggagaaggga	1440
acatgtgtaa aaagtcogag ggcaacatca tgtcccctac attggcagga cgcaatggag	1500
tcttctcctg gtcaccctgc agccgccagt atctacacaa atttctaagc accgctcaag	1560
ctatctgcct tgctgatcag ccaaagcctg tgaaggata caagtatcct gagaaattgc	1620
caggagaatt atatgatgca aacacacagt gcaagtggca gttcggagag aaagccaagc	1680
tctgcatgct ggactttaaa aaggacatct gtaaagccct gtggtgccat cgtattggaa	1740
ggaaatgtga gactaaattt atgccagcag cagaaggcac aatttgtggg catgacatgt	1800
ggtgccgggg aggacagtgt gtgaaatatg gtgatgaagg ccccaagccc acctatggcc	1860
actggtcggg ctggtcttct tgggtcccat gctccaggac ctgcggaggg ggagtatctc	1920
ataggagtgc cctctgcacc aaccccaagc catcgcatgg aggggaagttc tgtgagggtc	1980
ccactcgcac tctgaagctc tgcaacagtc agaaatgtcc ccgggacagt gttgacttcc	2040
gtgctgtcca gtgtgccgag cacaacagca gacgattcag agggcggcac tacaagtgga	2100
agccttacac tcaagtagaa gatcaggact tatgcaact ctactgtatc gcagaaggat	2160
ttgattttct cttttctttg tcaataaaag tcaaagatgg gactccatgc tcggaggata	2220
gcccgaatgt ttgtatagat gggatatgtg agagagttgg atgtgacaat gtccttgat	2280
ctgatgctgt tgaagacgtc tgtgggtgt gtacgggaa taactcagcc tgcacgattc	2340
acaggggtct ctacaccaag caccaccaca ccaaccagta ttatcacatg gtcaccattc	2400
cttctggagc ccggagtatc cgcattctatg aaatgaacgt ctctacctcc tacatttctg	2460
tgcgcaatgc ctcagaagg tactacctga atgggcaact gacctggac tggcccgcc	2520
ggtacaaaat ttcgggcaact actttcgact acagacggtc ctataatgag cccgagaact	2580
taatcgctac tggaccaacc aacgagacac tgattgtgga gctgctgtt cagggaaagg	2640
acccgggtgt tgctgggaa tactccatgc ctgcctggg gaccgagaag cagccccctg	2700

-continued

cccagcccag ctacacttgg gccatcgtgc gctctgagtg ctccgtgtcc tgcggagggg	2760
gacagatgac cgtgagagag ggtgctaca gagacctgaa gtttcaagta aatatgtcct	2820
tctgcaatcc caagacacga cctgtcacgg ggctgggtgcc ttgcaaagta tctgcctgtc	2880
ctcccagctg gtccgtgggg aactggagtg cctgcagtcg gacgtgtggc ggggggtgcc	2940
agagccgccc cgtgcagtg acacggcggg tgcactatga ctcgagacca gtcccggcca	3000
gcctgtgccc tcagcctgtc cctccagca ggcaggcctg caactctcag agctgcccac	3060
ctgcatggag cgcggggccc tgggcagagt gctcacacac ctgtgggaag ggggtgagga	3120
agcgggcagt ggctgtaag agcaccaacc cctcgggcag agcgcagctg ctgcccgaag	3180
ctgtctgcac ctccgagccc aagcccagga tgcctgaagc ctgtctgctt cagcgtgtcc	3240
acaagcccaa gaagctgcag tggctggtgt ccgctgtgtc ccagtgtctt gtgacatgtg	3300
aaagaggaac acagaaaaga ttcttaaaat gtgctgaaaa gtatgtttct ggaaagtatc	3360
gagagctggc ctcaaagaag tgctcacatt tgccgaagcc cagcctggag ctggaacgtg	3420
cctgcgcccc gcttccatgc cccaggcacc cccatttgc tgcgtcgagg ccctcgaggg	3480
gcagctggtt tgccacacc tggctcagc gcacggccag ctgtggggga ggcgttcaga	3540
cgaggtccgt gcagtgccgt gctgggggcc ggcgggcctc aggtgcctc ctgcaccaga	3600
agccttcggc ctccctggcc tgcaaacctc acttctgccc cattgcagag aagaaagatg	3660
ccttctgcaa agactacttc cactgggtgt acctggtacc ccagcacggg atgtgcagcc	3720
acaagtctta cggaagcag tgctgcaaga ctgtcttaa gtccaacttg tgagttggga	3780
ccgtctcccg tagcagagaa agtgctgtgc tggcacagaa atttccaca aatgagctgt	3840
gcaatctacg tcggaatata tccaaggaag agcaaaagca aaagaagaaa accgtgttag	3900
gctctttgac caggagtgtg tgtatgtgtt tcaactgtgag cctgggtgca gacctgtgtc	3960
cccatgcaca cagtgtctcc tgtcaggctg aaatgtggca cctgggcaga cagagctgtg	4020
gctcgtgagg cagaaggcag gc	4042

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding an amino acid sequence drawn from the group consisting of SEQ ID NOS: 2 and 4.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that:

(a) encodes the amino acid sequence shown in SEQ ID NO: 4; and

(b) hybridizes under stringent conditions of hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% SDS, 1mM EDT at 65° C. and washing in 0.1×SSC/1% SDS at 68°

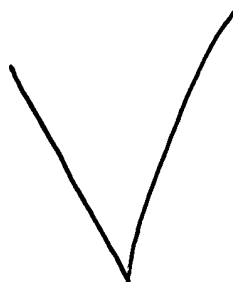
C. to the nucleotide sequence of SEQ ID NO: 3 or the complement thereof.

3. An isolated nucleic acid molecule according to claim 1 wherein said nucleotide sequence is present in cDNA.

4. An isolated nucleic acid molecule encoding the amino acid sequence presented in SEQ ID NO:4.

5. An isolated nucleic acid molecule encoding the amino acid sequence presented in SEQ ID NO:2.

* * * * *



This Page Blank (uspto)



US006444456B1

(12) **United States Patent**
Walke et al.(10) **Patent No.:** US 6,444,456 B1
(45) **Date of Patent:** Sep. 3, 2002(54) **HUMAN G-COUPLED PROTEIN RECEPTOR KINASES AND POLYNUCLEOTIDES ENCODING THE SAME**(75) **Inventors:** D. Wade Walke, Spring; Nathaniel L. Wilganowski, Houston; C. Alexander Turner, Jr., The Woodlands, all of TX (US)(73) **Assignee:** Lexicon Genetics Incorporated, The Woodlands, TX (US)(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.(21) **Appl. No.:** 09/802,117(22) **Filed:** Mar. 8, 2001**Related U.S. Application Data**(60) **Provisional application No.** 60/188,449, filed on Mar. 10, 2000.(51) **Int. Cl.⁷** C12N 9/12; C12N 15/79; C12N 5/10; C07H 21/04; C12Q 1/48(52) **U.S. Cl.** 435/194; 536/23.2; 435/15; 435/325; 435/252.3; 435/320.1(58) **Field of Search** 536/23.2; 435/15; 435/194, 325, 252.3, 320.1(56) **References Cited****U.S. PATENT DOCUMENTS**

4,215,051 A 7/1980 Schroeder et al.
 4,376,110 A 3/1983 David et al.
 4,594,595 A 6/1986 Struckman
 4,631,211 A 12/1986 Houghten
 4,689,405 A 8/1987 Frank et al.
 4,713,326 A 12/1987 Dattagupta et al.
 4,946,778 A 8/1990 Ladner et al.
 5,252,743 A 10/1993 Barrett et al.
 5,424,186 A 6/1995 Fodor et al.
 5,445,934 A 8/1995 Fodor et al.
 5,459,127 A 10/1995 Felgner et al.
 5,532,151 A 7/1996 Chantry et al.
 5,556,752 A 9/1996 Lockhart et al.
 5,591,618 A 1/1997 Chantry et al.
 5,700,637 A 12/1997 Southern
 5,744,305 A 4/1998 Fodor et al.
 5,830,721 A 11/1998 Stemmer et al.
 5,837,458 A 11/1998 Minshull et al.
 5,869,336 A 2/1999 Meyer et al.
 5,877,397 A 3/1999 Lonberg et al.
 5,948,767 A 9/1999 Scheule et al.
 6,075,181 A 6/2000 Kucherlapati et al.
 6,110,490 A 8/2000 Thierry
 6,150,584 A 11/2000 Kucherlapati et al.
 6,331,423 B1 * 12/2001 Guegler et al. 435/194

FOREIGN PATENT DOCUMENTS

WO WO 01/38503 A2 5/2001

OTHER PUBLICATIONS

Broun et al., Science 282:1315-1317, 1998.*
 Van de Loo et al., Proc. Natl. Acad. Sci. 92:6743-6747, 1995.*
 Weiss et al., GenEMBL accession No. AF063016, Dec. 1998.*
 Weiss et al., SPTREMBL accession No. Q9Z2G7, May 1999.*
 Database EMBL (Online) May 7, 2000, Birren, B. et al. "Homo sapiens chromosome 17, clone RP11-144C9", Database accession No. AC068693, XP002181811.
 Weiss, Ellen R. et al., "The cloning of GRK7, a candidate cone opsin kinase, from cone and rod-dominant mammalian retinas," Molecular Vision, vol. 4, Dec. 8, 1998, p. 27, XP002181808.
 Premont, Richard T. et al., "The GRK4 subfamily of G protein-coupled receptor kinases: Alternative splicing, gene organization, and sequence conservation," Journal of Biological Chemistry, vol. 274, No. 41, Oct. 8, 1999, pp. 29381-29389, XP002181809.
 Benovic, Jeffrey L. et al., "Molecular cloning and expression of GRK6: A new member of the G protein-coupled receptor kinase family," Journal of Biological Chemistry, vol. 268, No. 26, 1993, pp. 19521-19527, XP002181810.
 International Search Report, International Application No. PCT/US01/07500, Nov. 8, 2001.
 Bird et al, 1988, "Single-Chain Antigen-Binding Proteins", Science 242:423-426.
 Bitter et al, 1987, "Expression and Secretion Vectors for Yeast", Methods in Enzymology 153:516-544.
 Colbere-Garapin et al, 1981, "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol. 150:1-14.
 Gautier et al, 1987, "α-DNA IV: α-anomeric and β-anomeric tetrahydropyrimidylates covalently linked to intercalating oxazolopyridocarbazole. Synthesis, physicochemical properties and poly (rA) binding", Nucleic Acids Research 15(16):6625-6641.
 Greenspan et al, 1993, "Idiotypes: structure and immunogenicity", FASEB Journal 7:437-444.
 Huse et al, 1989, "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", Science 246:1275-1281.
 Huston et al, 1988, "Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *escherichia coli*", Proc. Natl. Acad. Sci. USA 85:5879-5883.
 Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and RNase H", FEBS Letters 215(2):327-330.

(List continued on next page.)

Primary Examiner—Ponnathapu Achutamurthy
Assistant Examiner—Delia Ramirez(57) **ABSTRACT**

Novel human polynucleotide and polypeptide sequences are disclosed that can be used in therapeutic, diagnostic, and pharmacogenomic applications.

4 Claims, No Drawings

OTHER PUBLICATIONS

- Inoue et al, 1987, "Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides", *Nucleic Acids Research* 15(15):6131-6149.
- Inouye & Inouye, 1985, "Up-promoter mutations in the *lpp* gene of *Escherichia coli*", *Nucleic Acids Research* 13(9):3101-3110.
- Janknecht et al, 1991, "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus", *PNAS* 88:8972-8976.
- Kohler & Milstein, 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity", *Nature* 256:495-497.
- Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection", *Proc. Natl. Acad. Sci. USA* 81:3655-3659.
- Lowy et al, 1980, "Isolation of Transforming DNA: Cloning the Hamster *aprt* Gene", *Cell* 22:817-823.
- Morrison et al, 1984, "Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains", *Proc. Natl. Acad. Sci. USA* 81:6851-6855.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", *Proc. Natl. Acad. Sci. USA* 78(4):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies possessing novel effector functions", *Nature* 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", *J. of Immunology* 147:2429-2438.
- O'Hare et al, 1981, "Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase", *Proc. Natl. Acad. Sci. USA* 78(3):1527-1531.
- Ruther et al, 1983, "Easy identification of cDNA clones", *EMBO Journal* 2(10):1791-1794.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", *Gene* 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", *Proc. Natl. Acad. Sci. USA* 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", *J. Virol.* 46(2):584-593.
- Stein et al, 1988, "Physicochemical properties of phosphorothioate oligodeoxynucleotides", *Nucleic Acids Research* 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", *Proc. Natl. Acad. Sci. USA* 48:2026-2034.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", *Nature* 314:452-454.
- Van Heeke et al, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", *J. Biol. Chemistry* 264(10):5503-5509.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", *Nature* 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", *Cell* 11:223-232.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", *Proc. Natl. Acad. Sci. USA* 77(6):3567-3570.

* cited by examiner

1

HUMAN G-COUPLED PROTEIN RECEPTOR KINASES AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application No. 60/188,449 which was filed on Mar. 10, 2000 and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding proteins that share sequence similarity with animal kinases. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or over express the disclosed polynucleotides, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed polynucleotides that can be used for diagnosis, drug screening, clinical trial monitoring and the treatment of diseases and physiological disorders.

2. BACKGROUND OF THE INVENTION

Kinases mediate phosphorylation of a wide variety of proteins and compounds in the cell. In conjunction with phosphatases, kinases are involved in a range of regulatory and signaling pathways. Given the physiological importance of kinases, they have been subject to intense scrutiny and are proven drug targets.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal kinases, including, but not limited to G-protein coupled receptor kinases (GRKs). As such, the novel polynucleotides encode novel GRKs having homologues and orthologues across a range of phyla and species.

The novel human polynucleotides described herein, encode open reading frames (ORFs) encoding proteins of 553 and 353 amino acids in length (see SEQ ID NOS:2 and 4 respectively).

The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof, that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHP polynucleotides (e.g., expression constructs that place the described polynucleotide under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" (which can be conditional) that do not express a functional NHP. Knock-out mice can be produced in several ways, one of which involves the use of mouse embryonic stem cells ("ES cells") lines that contain gene trap mutations in a murine homolog of at least one of the described NHPs. When the unique NHP sequences described in SEQ ID NOS:1-5 are "knocked-out"

2

they provide a method of identifying phenotypic expression of the particular gene as well as a method of assigning function to previously unknown genes. Additionally, the unique NHP sequences described in SEQ ID NOS:1-5 are useful for the identification of coding sequence and the mapping a unique gene to a particular chromosome.

Further, the present invention also relates to processes for identifying compounds that modulate, i.e., act as agonists or antagonists, of NHP expression and/or NHP activity that utilize purified preparations of the described NHPs and/or NHP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequence of the novel human ORFs encoding the described novel human kinase proteins. SEQ ID NO:5 describes a full length ORF and flanking regions.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPs, described for the first time herein, are novel proteins that are expressed in, inter alia, human cell lines, and human fetal brain, adult brain, pituitary, cerebellum, spinal cord, thymus, kidney, fetal liver, prostate, testis, adrenal gland, small intestine, skeletal muscle, uterus, placenta, mammary gland, and pericardium cells. The described sequences were compiled from gene trapped sequences in conjunction with sequences available in GENBANK, and cDNAs from adrenal gland, skeletal muscle, thymus, and testis libraries (Edge Biosystems, Gaithersburg, Md.).

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described polynucleotides, including the specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of an NHP that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of a NHP, or one of its domains (e.g., a receptor/ligand binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing. As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly

stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1× SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2× SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, supra), yet still encode a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar to corresponding regions of SEQ ID NO:1 (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using default parameters).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP encoding polynucleotides. Such hybridization conditions can be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc.

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-5 can be used as a hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics, polymers, metal or metallized substrates, crystalline or polycrystalline substrates, etc.). Of particular note are spatially addressable arrays (i.e., gene chips, microtiter plates, etc.) of oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-5, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, inter alia, U.S. Pat. Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-5 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-5.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-5 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

Probes consisting of sequences first disclosed in SEQ ID NOS:1-5 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

As an example of utility, the sequences first disclosed in SEQ ID NOS:1-5 can be utilized in microarrays or other assay formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences first disclosed in SEQ ID NOS:1-5 in silico and by comparing previously collected genetic databases and the disclosed sequences using computer software known to those in the art.

Thus the sequences first disclosed in SEQ ID NOS:1-5 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific oligonucleotide sequence(s) first disclosed in the SEQ ID

NOS: 1-5. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (e.g., the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, Mich., etc.), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6× SSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences can be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation, or as NHP regulating aptamers.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids

Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue known or suspected to express an allele of a NHP gene.

The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase

H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, immune disorders, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, N.Y.)

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, alkaline phosphatase-NHP or NHP-alkaline phosphatase fusion proteins. In cases where a NHP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

An additional application of the described novel human polynucleotide sequences is their use in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Pat. Nos. 5,830,721 and 5,837,458 which are herein incorporated by reference in their entirety.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Pat. No. 5,869, 336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

Where, as in the present instance, some of the described NHP peptides or polypeptides are thought to be cytoplasmic proteins, expression systems can be engineered that produce soluble derivatives of a NHP (corresponding to a NHP extracellular and/or intracellular domains, or truncated polypeptides lacking one or more hydrophobic domains) and/or NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP domain to an IgFc), NHP antibodies, and anti-idiotypic antibodies (including Fab fragments) that can be used in therapeutic applications. Preferably, the above expression systems are engineered to allow the desired peptide or polypeptide to be recovered from the culture media.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHP, as well as compounds or nucleotide constructs that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of a NHP in the body. The use of engineered host cells and/or animals can offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor/ligand of a NHP, but can also identify compounds that trigger NHP-mediated activities or pathways.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains corresponding to NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, i.e., fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively antagonize the endogenous NHP or a protein interactive therewith. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 The NHP Sequences

The cDNA sequences and corresponding deduced amino acid sequences of the described NHPs are presented in the Sequence Listing.

Expression analysis has provided evidence that the described NHPs can be expressed in human tissues as well as gene trapped human cells. In addition to GRKs, the described NHPs share significant similarity to a range of kinase families from a variety of phyla and species. Similar polynucleotides encoding GRK proteins, as well as uses and applications that are germane to the described NHPs are described in U.S. Pat. Nos. 5,591,618 and 5,532,151 which are herein incorporated by reference in their entirety.

5.2 NHPS and NHP Polypeptides

NHP products, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include, but are not limited to, the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to the NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP-encoding polynucleotides. The NHPs display initiator methionines in a DNA sequence context consistent with eucaryotic translation initiation site, and a weak signal sequence characteristic of membrane associated proteins.

The NHP amino acid sequences of the invention include the amino acid sequences presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degen-

erate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and modify a NHP substrate, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by a NHP nucleotide sequence described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where the NHP peptide or polypeptide can exist, or has been engineered to exist, as a soluble or secreted molecule, the soluble NHP peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, *in situ*. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3)

harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign polynucleotides. The virus grows in *Spodoptera frugiperda* cells. A NHP encoding polynucleotide sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted polynucleotide is expressed (e.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the

entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp⁺ or apr⁺ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg⁺, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the polynucleotide of interest is subcloned into a

vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Also encompassed by the present invention are fusion proteins that direct the NHP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHPs to antibody molecules or their Fab fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHP would also transport the NHP to the desired location within the cell. Alternatively targeting of NHP or its nucleic acid sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in *Liposomes: A Practical Approach*, New, RRC ed., Oxford University Press, New York and in U.S. Pat. Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective disclosures which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHP to the target site or desired organ, where they cross the cell membrane and/or the nucleus where the NHP can exert its functional activity. This goal may be achieved by coupling of the NHP to a cytokine or other ligand that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. application Ser. No. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes and can optionally be engineered to include nuclear localization sequences.

5.3 Antibodies to NHP Products

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab)'_2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention can be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction with gene therapy to, for example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with the NHP, a NHP peptide (e.g., one corresponding to a functional domain of a NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats,

and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mabs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger et al., 1984, *Nature*, 312:604-608; Takeda et al., 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Pat. Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety. Also encompassed by the present invention is the use of fully humanized monoclonal antibodies as described in U.S. Pat. No. 6,150,584 and respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, *Science* 242:423-426; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 341:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab)'_2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab)'_2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, *Science*, 246:1275-1281) to

allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor/ligand can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind, activate, or neutralize a NHP, NHP receptor, or NHP ligand. Such anti-idiotypic

antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHP mediated pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 5

<210> SEQ ID NO 1

<211> LENGTH: 1662

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```

atggtggaca tgggggcctt ggayaacctg atcgccaaca cgcctacct gcaggcccg 60
aagccctcgg actgcgacag caaagagctg cagcggcggc gccgtagcct ggccctgcc 120
gggtgcagg gctgcgcgga gctccgccag aagctgtccc tgaacttcca cagcctgtgt 180
gagcagcagc ccatcggtcg cgcctcttc cgtgacttcc tagccacagt gccacgttc 240
cgcaaggcgg caaccttctt agaggacgtg cagaactggg agctggccga ggagggaccc 300
accaaagaca gcgcgctgca ggggctgggt gccacttgtg cagtgcccc tgccccggg 360
aaccgcgaac cttctctcag ccaggccgtg gccaccaagt gccaaagcag caccactgag 420
gaagagcgag tggctgcagt gacgctggcc aaggctgagg ccatggcttt cttgcaagag 480
cagcccttta aggatctcgt gaccagcgcc ttctacgaca agtttctgca gtggaaactc 540
ttcgagatgc aaccagtgtc agacaagtac ttactgagt tcagagtgtc ggggaaaggt 600
ggttttgggg aggtatgtgc cgtccagggt aaaaacactg ggaagatgta tgcctgtaag 660
aaactggaca agaagcggct gaagaagaaa ggtggcgaga agatggctct cttggaaaa 720
gaaatcttgg agaaggtcag cagcccttcc attgtctctc tggcctatgc ctttgagagc 780
aagaccatc tctgccttgt catgagcctg atgaatgggg gagacctcaa gttccacatc 840
tacaacgtgg gcacgcgtgg cctggacatg agccgggtga tcttttactc ggcccagata 900
gcctgtggga tgctgcacct ccatgaactc ggcatcgtct atcgggacat gaagcctgag 960
aatgtgcttc tggatgacct cggaactgc aggttatctg acctggggct ggccgtggag 1020
atgaagggtg gcaagcccat caccagagg gctggaacca atggttacat ggctcctgag 1080
atcctaattg aaaaggtaag ttattcctat cctgtggact ggtttgcat gggatgcagc 1140
atztatgaaa tggttgtctg acgaacacca ttcaaagatt acaaggaaaa ggtcagtaaa 1200
gaggatctga agcaaagaac tctgcaagac gaggtcaaat tccagcatga taacttcaca 1260
gaggaagcaa aagatatttg caggctcttc ttggctaaga aaccagagca acgcttagga 1320
agcagagaaa agtctgatga tcccaggaaa catcatttct ttaaaacgat caactttcct 1380
cgcctggaa gctggcctaat tgaaccccca tttgtccag acccttcagt ggtttatgcc 1440
aaagacatcg ctgaaattga tgatttctct gaggttcggg ggtggaatt tgatgacaaa 1500

```

-continued

gataagcagt tcttcaaaaa ctttgcgaca ggtgctgttc ctatagcatg gcaggaagaa 1560
 attatagaaa cgggactgtt tgaggaactg aatgacccca acagacctac gggttgtgag 1620
 gagggtaatt catccaagtc tggcgtgtgt ttgttattgt aa 1662

<210> SEQ ID NO 2
 <211> LENGTH: 553
 <212> TYPE: PRT
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

Met Val Asp Met Gly Ala Leu Asp Asn Leu Ile Ala Asn Thr Ala Tyr
 1 5 10 15
 Leu Gln Ala Arg Lys Pro Ser Asp Cys Asp Ser Lys Glu Leu Gln Arg
 20 25 30
 Arg Arg Arg Ser Leu Ala Leu Pro Gly Leu Gln Gly Cys Ala Glu Leu
 35 40 45
 Arg Gln Lys Leu Ser Leu Asn Phe His Ser Leu Cys Glu Gln Gln Pro
 50 55 60
 Ile Gly Arg Arg Leu Phe Arg Asp Phe Leu Ala Thr Val Pro Thr Phe
 65 70 75 80
 Arg Lys Ala Ala Thr Phe Leu Glu Asp Val Gln Asn Trp Glu Leu Ala
 85 90 95
 Glu Glu Gly Pro Thr Lys Asp Ser Ala Leu Gln Gly Leu Val Ala Thr
 100 105 110
 Cys Ala Ser Ala Pro Ala Pro Gly Asn Pro Gln Pro Phe Leu Ser Gln
 115 120 125
 Ala Val Ala Thr Lys Cys Gln Ala Ala Thr Thr Glu Glu Glu Arg Val
 130 135 140
 Ala Ala Val Thr Leu Ala Lys Ala Glu Ala Met Ala Phe Leu Gln Glu
 145 150 155 160
 Gln Pro Phe Lys Asp Phe Val Thr Ser Ala Phe Tyr Asp Lys Phe Leu
 165 170 175
 Gln Trp Lys Leu Phe Glu Met Gln Pro Val Ser Asp Lys Tyr Phe Thr
 180 185 190
 Glu Phe Arg Val Leu Gly Lys Gly Gly Phe Gly Glu Val Cys Ala Val
 195 200 205
 Gln Val Lys Asn Thr Gly Lys Met Tyr Ala Cys Lys Lys Leu Asp Lys
 210 215 220
 Lys Arg Leu Lys Lys Lys Gly Gly Glu Lys Met Ala Leu Leu Glu Lys
 225 230 235 240
 Glu Ile Leu Glu Lys Val Ser Ser Pro Phe Ile Val Ser Leu Ala Tyr
 245 250 255
 Ala Phe Glu Ser Lys Thr His Leu Cys Leu Val Met Ser Leu Met Asn
 260 265 270
 Gly Gly Asp Leu Lys Phe His Ile Tyr Asn Val Gly Thr Arg Gly Leu
 275 280 285
 Asp Met Ser Arg Val Ile Phe Tyr Ser Ala Gln Ile Ala Cys Gly Met
 290 295 300
 Leu His Leu His Glu Leu Gly Ile Val Tyr Arg Asp Met Lys Pro Glu
 305 310 315 320
 Asn Val Leu Leu Asp Asp Leu Gly Asn Cys Arg Leu Ser Asp Leu Gly
 325 330 335
 Leu Ala Val Glu Met Lys Gly Gly Lys Pro Ile Thr Gln Arg Ala Gly

-continued

340	345	350
Thr Asn Gly Tyr Met Ala Pro Glu Ile Leu Met Glu Lys Val Ser Tyr 355	360	365
Ser Tyr Pro Val Asp Trp Phe Ala Met Gly Cys Ser Ile Tyr Glu Met 370	375	380
Val Ala Gly Arg Thr Pro Phe Lys Asp Tyr Lys Glu Lys Val Ser Lys 385	390	395
Glu Asp Leu Lys Gln Arg Thr Leu Gln Asp Glu Val Lys Phe Gln His 405	410	415
Asp Asn Phe Thr Glu Glu Ala Lys Asp Ile Cys Arg Leu Phe Leu Ala 420	425	430
Lys Lys Pro Glu Gln Arg Leu Gly Ser Arg Glu Lys Ser Asp Asp Pro 435	440	445
Arg Lys His His Phe Phe Lys Thr Ile Asn Phe Pro Arg Leu Glu Ala 450	455	460
Gly Leu Ile Glu Pro Pro Phe Val Pro Asp Pro Ser Val Val Tyr Ala 465	470	475
Lys Asp Ile Ala Glu Ile Asp Asp Phe Ser Glu Val Arg Gly Val Glu 485	490	495
Phe Asp Asp Lys Asp Lys Gln Phe Phe Lys Asn Phe Ala Thr Gly Ala 500	505	510
Val Pro Ile Ala Trp Gln Glu Glu Ile Ile Glu Thr Gly Leu Phe Glu 515	520	525
Glu Leu Asn Asp Pro Asn Arg Pro Thr Gly Cys Glu Glu Gly Asn Ser 530	535	540
Ser Lys Ser Gly Val Cys Leu Leu Leu 545	550	

<210> SEQ ID NO 3
 <211> LENGTH: 1062
 <212> TYPE: DNA
 <213> ORGANISM: homo sapiens

<400> SEQUENCE: 3

atgggtggaca tggggggccct ggacaaacctg atcgccaaca ccgcctacct gcaggcccgg	60
aagccctcgg actgcgacag caaagagctg cagcggcggc gccgtagcct ggcctgccc	120
gggctgcagg gctgcgcgga gctccgccag aagctgtccc tgaacttcca cagcctgtgt	180
gagcagcagc ccatcggtcg ccgcctcttc cgtgacttcc tagccacagt gccacgttc	240
cgcaaggcgg caaccttctt agaggacgtg cagaactggg agctggccga ggagggaacc	300
accaaagaca gcgcgctgca ggggctggtg gccacttggt cgagtgcgcc tgccccgggg	360
aaccgcgaac ctttcctcag ccaggccgtg gccaccaagt gccaaagcag caccactgag	420
gaagagcgag tggctgcagt gacgctggcc aaggctgagg ccatggcttt cttgcaagag	480
cagcccttta aggtattcgt gaccagcgcc ttctacgaca agttctgtca gtggaaactc	540
ttcgagatgc aaccagtgtc agacaagtac ttactgagt tcagagtgtc ggggaaaggt	600
ggttttgggg aggtatgtgc cgtccagggtg aaaaacactg ggaagatgta tgctgttaag	660
aaactggaca agaagcggct gaagaagaaa ggtggcgaga agatggctct cttggaaaag	720
gaaatcttgg agaaggtcag cagcccttcc attgtctctc tggcctatgc ctttgagagc	780
aagaccatc tctgccttgt catgagcctg atgaatgggg gagacctcaa gttccacatc	840
tacaacgtgg gcacgcgtgg cctggacatg agccgggtga tcttttactc ggcccagata	900

-continued

```

gctgtgtggga tgctgcacct ccatgaactc ggcacgtct atcgggacat gaagcctgag 960
aatgtgcttc tggatgacct cggcaactgc aggttatctg acctggggct ggccgtggag 1020
atgaaggggtg gcaagcccat caccagagg agaaaagtct ga 1062

```

```

<210> SEQ ID NO 4
<211> LENGTH: 353
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

```

```

<400> SEQUENCE: 4

```

```

Met Val Asp Met Gly Ala Leu Asp Asn Leu Ile Ala Asn Thr Ala Tyr
 1             5             10            15
Leu Gln Ala Arg Lys Pro Ser Asp Cys Asp Ser Lys Glu Leu Gln Arg
          20             25            30
Arg Arg Arg Ser Leu Ala Leu Pro Gly Leu Gln Gly Cys Ala Glu Leu
      35             40            45
Arg Gln Lys Leu Ser Leu Asn Phe His Ser Leu Cys Glu Gln Gln Pro
 50             55            60
Ile Gly Arg Arg Leu Phe Arg Asp Phe Leu Ala Thr Val Pro Thr Phe
65             70            75            80
Arg Lys Ala Ala Thr Phe Leu Glu Asp Val Gln Asn Trp Glu Leu Ala
          85             90            95
Glu Glu Gly Pro Thr Lys Asp Ser Ala Leu Gln Gly Leu Val Ala Thr
      100            105           110
Cys Ala Ser Ala Pro Ala Pro Gly Asn Pro Gln Pro Phe Leu Ser Gln
      115            120           125
Ala Val Ala Thr Lys Cys Gln Ala Ala Thr Thr Glu Glu Glu Arg Val
      130            135           140
Ala Ala Val Thr Leu Ala Lys Ala Glu Ala Met Ala Phe Leu Gln Glu
      145            150           155           160
Gln Pro Phe Lys Asp Phe Val Thr Ser Ala Phe Tyr Asp Lys Phe Leu
          165            170           175
Gln Trp Lys Leu Phe Glu Met Gln Pro Val Ser Asp Lys Tyr Phe Thr
      180            185           190
Glu Phe Arg Val Leu Gly Lys Gly Gly Phe Gly Glu Val Cys Ala Val
      195            200           205
Gln Val Lys Asn Thr Gly Lys Met Tyr Ala Cys Lys Lys Leu Asp Lys
      210            215           220
Lys Arg Leu Lys Lys Lys Gly Gly Glu Lys Met Ala Leu Leu Glu Lys
      225            230           235           240
Glu Ile Leu Glu Lys Val Ser Ser Pro Phe Ile Val Ser Leu Ala Tyr
          245            250           255
Ala Phe Glu Ser Lys Thr His Leu Cys Leu Val Met Ser Leu Met Asn
          260            265           270
Gly Gly Asp Leu Lys Phe His Ile Tyr Asn Val Gly Thr Arg Gly Leu
          275            280           285
Asp Met Ser Arg Val Ile Phe Tyr Ser Ala Gln Ile Ala Cys Gly Met
          290            295           300
Leu His Leu His Glu Leu Gly Ile Val Tyr Arg Asp Met Lys Pro Glu
      305            310           315           320
Asn Val Leu Leu Asp Asp Leu Gly Asn Cys Arg Leu Ser Asp Leu Gly
          325            330           335
Leu Ala Val Glu Met Lys Gly Gly Lys Pro Ile Thr Gln Arg Arg Lys
          340            345           350

```

-continued

Val

<210> SEQ ID NO 5
<211> LENGTH: 2249
<212> TYPE: DNA
<213> ORGANISM: homo sapiens

<400> SEQUENCE: 5

```
aaaactgctc tgagggcatc atgctttgag gaagcccagg aggaaacact gcagagaggc 60
tcaaccaccc cagctctccc agctgagctc agccaccac cagatcccca gctgaatgca 120
accataagag tgagtcacag ttctaccctg ctaggctgcc accacattcc taagaaccac 180
gggaaaaggc atttgcctct ccgaagaaat tctcagactg atttttctact gtattgtcag 240
gccacaggac tcaactgtaaa tcccttggac gttgtctcac ccgggaaggg aaagcagcca 300
gcagccctcc agccctcttg tgctttccct gggagtgcgc ccctgctca gccatgggtg 360
acatgggggc cctggacaac ctgatcgcca acaccgcta cctgcaggcc cggaagccct 420
cggactgcga cagcaaagag ctgcagcggc ggcggcgtag cctggccctg cccgggctgc 480
agggtgcgc ggagctccgc cagaagctgt ccctgaactt ccacagcctg tgtgagcagc 540
agcccatcgg tcgccgcctc ttccgtgact tctagccac agtgcccacg ttccgcaagg 600
cggcaacctt cctagaggac gtgcagaact gggagctggc cgaggaggga cccaccaaag 660
acagcgcgct gcaggggctg gtggccactt gtgcgagtgc ccctgccccg gggaaaccgc 720
aacccttcct cagccaggcc gtggccacca agtgccaagc agccaccact gaggaagagc 780
gagtggctgc agtgacgctg gccaaggctg aggcctatgg tttcttgcaa gacagccct 840
ttaaggattt cgtgaccagc gccttctacg acaagtttct gcagtggaaa ctcttcgaga 900
tgcaaccagt gtcagacaag tacttctactg agttcagagt gctggggaaa ggtggttttg 960
gggaggtatg tgccgtccag gtgaaaaaca ctgggaagat gtatgcctgt aagaaactgg 1020
acaagaagcg gctgaagaag aaaggtggcg agaagatggc tctcttgcaa aaggaaatct 1080
tggagaaggt cagcagccct ttcattgtct ctctggccta tgcccttgag agcaagaccc 1140
atctctgcct tgtcatgagc ctgatgaatg ggggagacct caagtccac atctacaacg 1200
tgggcacgcg tggcctggac atgagccggg tgatctttta ctcgcccgag atagcctgtg 1260
ggatgctgca cctccatgaa ctcggcacg tctatcgga catgaagcct gagaatgtgc 1320
ttctggatga cctcggaac tgcaggttat ctgacctggg gctggccgtg gagatgaagg 1380
gtggcaagcc catcacccag agggctggaa ccaatggta catggctcct gagatcctaa 1440
tggaaaagggt aagttattcc tatcctgtgg actggtttgc catgggatgc agcatttatg 1500
aaatggttgc tggacgaaca ccattcaaag attacaagga aaaggtcagt aaagaggatc 1560
tgaagcaaag aactctgcaa gacgaggtca aattccagca tgataacttc acagagggaag 1620
caaaagatat ttgcaggctc ttcttgcta agaaaccaga gcaacgctta ggaagcagag 1680
aaaagtctga tgatccagg aaacatcatt tctttaaac gatcaacttt cctcgccctg 1740
aagctggcct aattgaaccc ccattttgtc cagacccttc agtggtttat gccaaagaca 1800
tcgctgaaat tgatgatttc tctgaggttc ggggggtgga atttgatgac aaagataagc 1860
agttcttcaa aaactttgcg acaggtgctg ttccctatagc atggcaggaa gaaattatag 1920
aaacgggact gtttgaggaa ctgaatgacc ccaacagacc tacgggttgt gagggggta 1980
attcatccaa gtctggcggtg tgtttgttat tgtaaattgc tctctttacc agacaggcag 2040
```

-continued

caggagtctc ggctgacata atcctcgaat gttccacacg tggaaatctg tggaatgagg	2100
gctaatacagt taggagggac atcacacaacca caaaacaatt caaaagacag gcaagctcac	2160
tactagaaca cattttattt tttttttctt ttttcataaa gatgagtaaa gtctcagttt	2220
tcactgaggg cagggaaaag gaacactca	2249

What is claimed is:

1. An isolated nucleic acid molecule comprising the nucleotide sequence disclosed in SEQ ID NO: 1.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that:

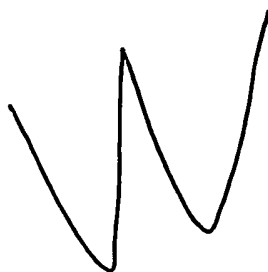
(a) encodes the amino acid sequence shown in SEQ ID NO: 2; and

(b) hybridizes under highly stringent conditions to the polynucleotide sequence of SEQ ID NO: 1 or the complete complement thereof.

3. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO:2.

4. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO:4.

* * * * *



This Page Blank (uspto)



US006444153B1

(12) **United States Patent**
Shah et al.

(10) **Patent No.:** **US 6,444,153 B1**
(45) **Date of Patent:** **Sep. 3, 2002**

(54) **IN-LINE COMPOUNDING/EXTRUSION
DEPOSITION AND MOLDING APPARATUS
AND METHOD OF USING THE SAME**

(75) Inventors: **Suresh Deepchand Shah, Troy; Jason
Alan Walte, Lake Orion, both of MI
(US)**

(73) Assignee: **Delphi Technologies, Inc., Troy, MI
(US)**

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/473,279**

(22) Filed: **Dec. 28, 1999**

(51) Int. Cl.⁷ **B29C 47/38**

(52) U.S. Cl. **264/211.21; 264/322**

(58) Field of Search **264/211.21, 211.22,
264/171.11, 210.2; 322; 425/114, 113, 121,
126.1**

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,925,381 A * 5/1990 Aoki et al.
5,000,397 A * 3/1991 Darrieux
5,185,117 A * 2/1993 Hawley
5,358,680 A * 10/1994 Boissonnat et al.
5,401,154 A * 3/1995 Sargent
6,126,433 A * 10/2000 Svoboda
6,186,769 B1 * 2/2001 Hawley

6,190,586 B1 * 2/2001 Abrams et al.

* cited by examiner

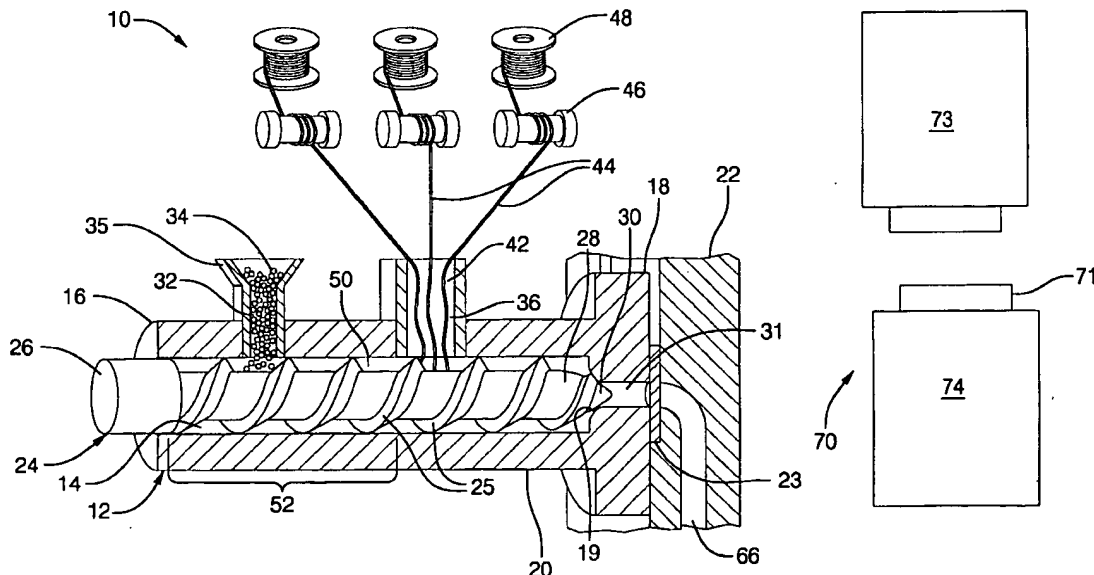
Primary Examiner—Mark Eashoo

(74) *Attorney, Agent, or Firm*—Kathryn A. Marra

(57) **ABSTRACT**

According to the present invention an in-line compounding/extrusion deposition and molding apparatus and method of using the same are provided. The apparatus comprises a single step compounding and extrusion apparatus which includes an extruder screw. The apparatus includes a first zone, a second zone, and a third zone. The first zone is used to melt an inlet material before the screw advances the melted inlet material into the second zone which comprises a preparation and cutting zone. Simultaneously, as the inlet material is melted in the first zone, the screw rotation feeds a reinforcing fiber bundle into the second zone where the reinforcing fiber bundle is prepared for melt impregnation and is sheared to a desired length. While in the second zone, mixing begins between the melted inlet material and the sheared reinforcing fiber bundle. Next the mixture is advanced into the third zone for uniform distributive mixing and impregnation of the sheared reinforcing fiber bundle with the melted inlet material to form a fiber bundle filled melt. The apparatus includes at least one winding/unwinding reel which continuously ensures that the reinforcing fiber bundle is under constant tension (no sagging or breaking thereof) during the X, Y, Z movement of the apparatus during melt deposition as well as during forward movement of the screw within the barrel.

16 Claims, 5 Drawing Sheets



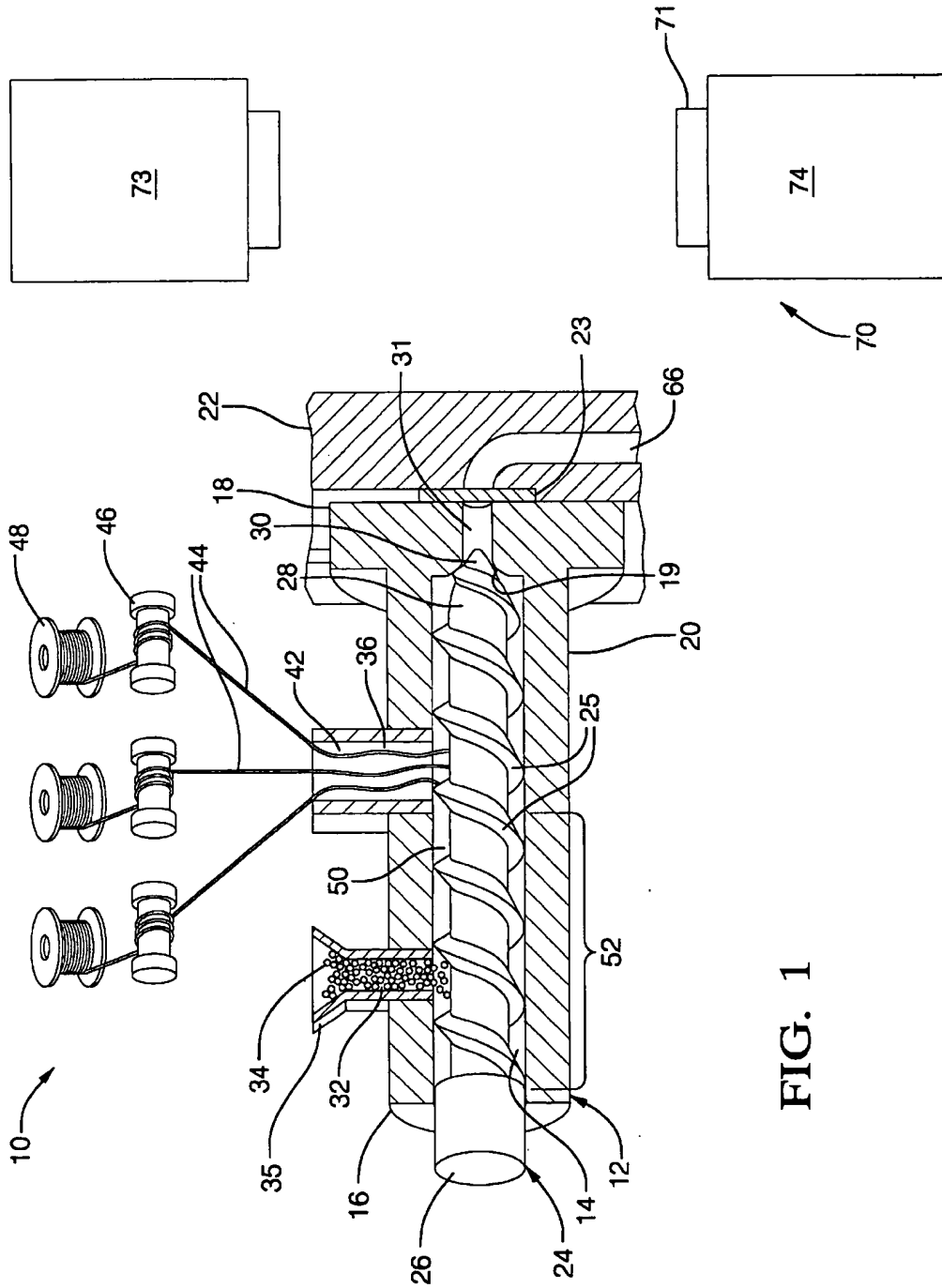


FIG. 1

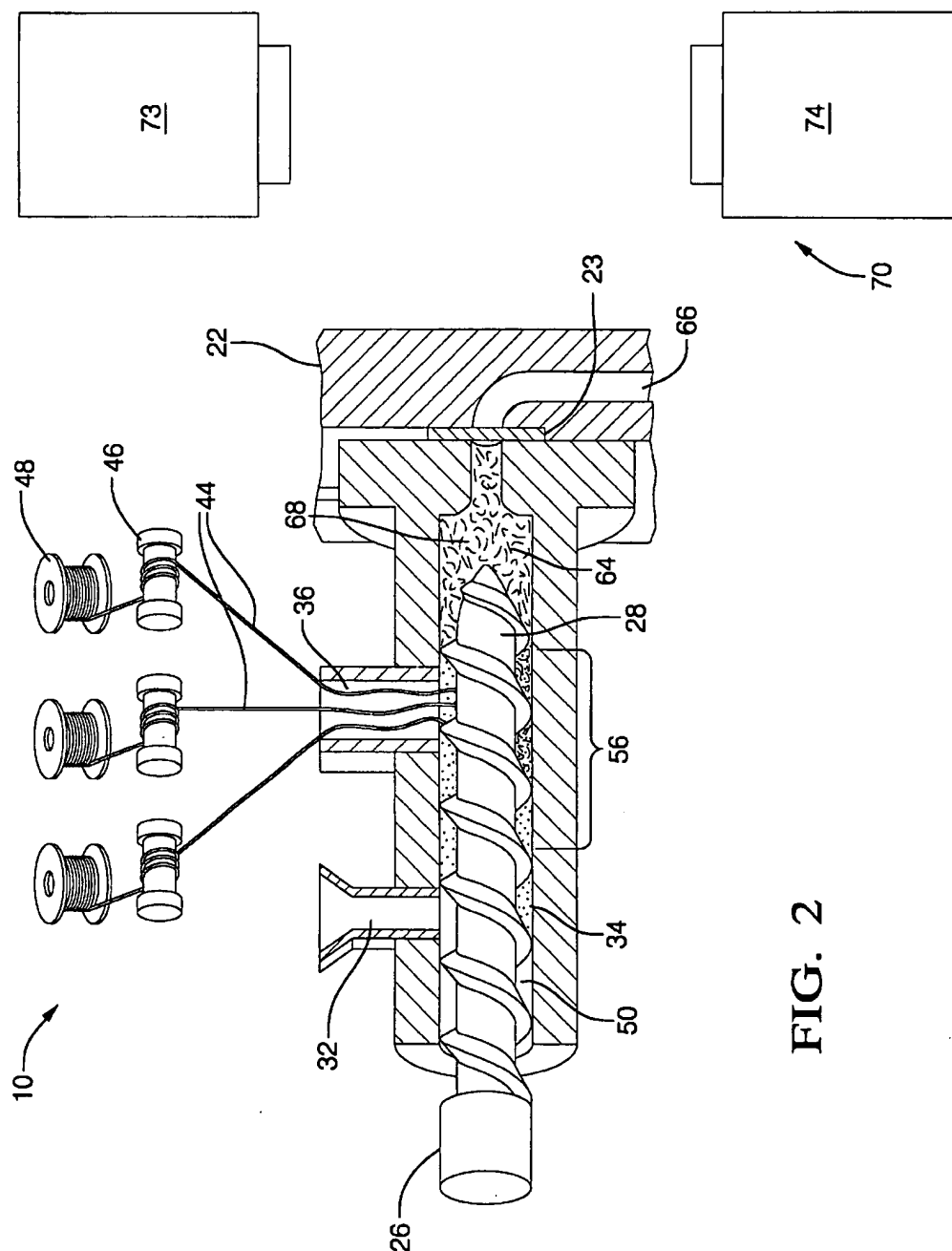


FIG. 2

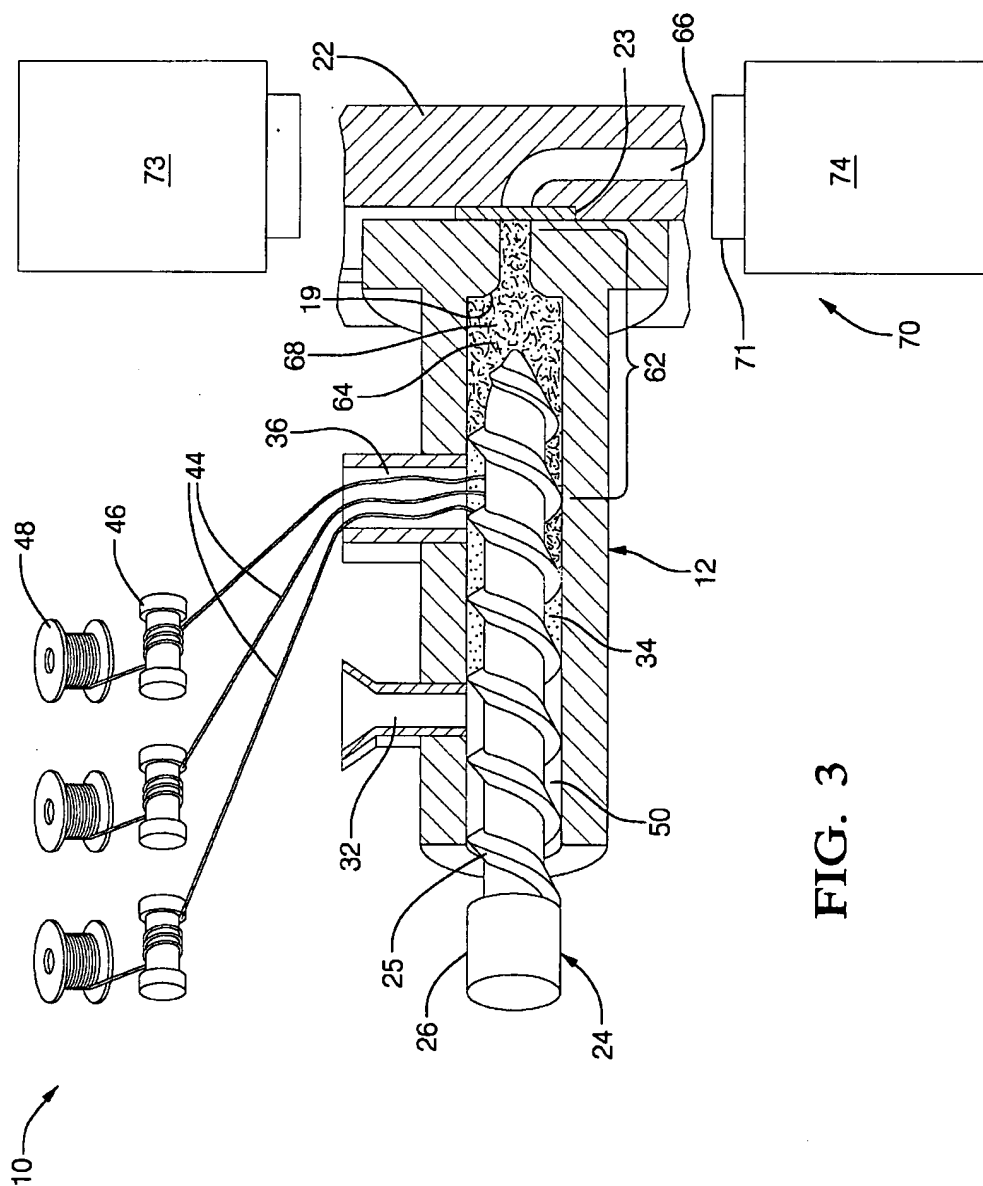


FIG. 3

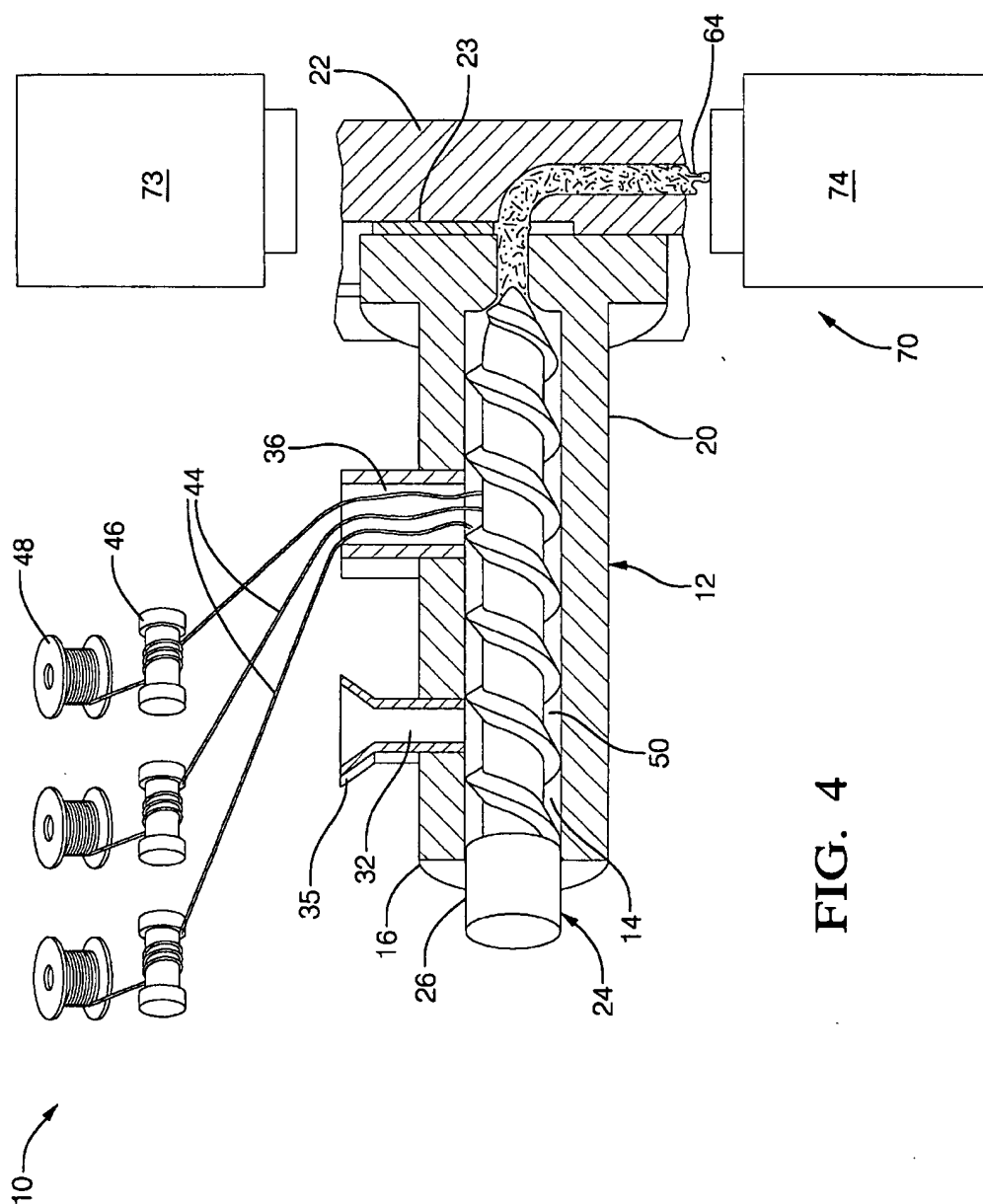


FIG. 4

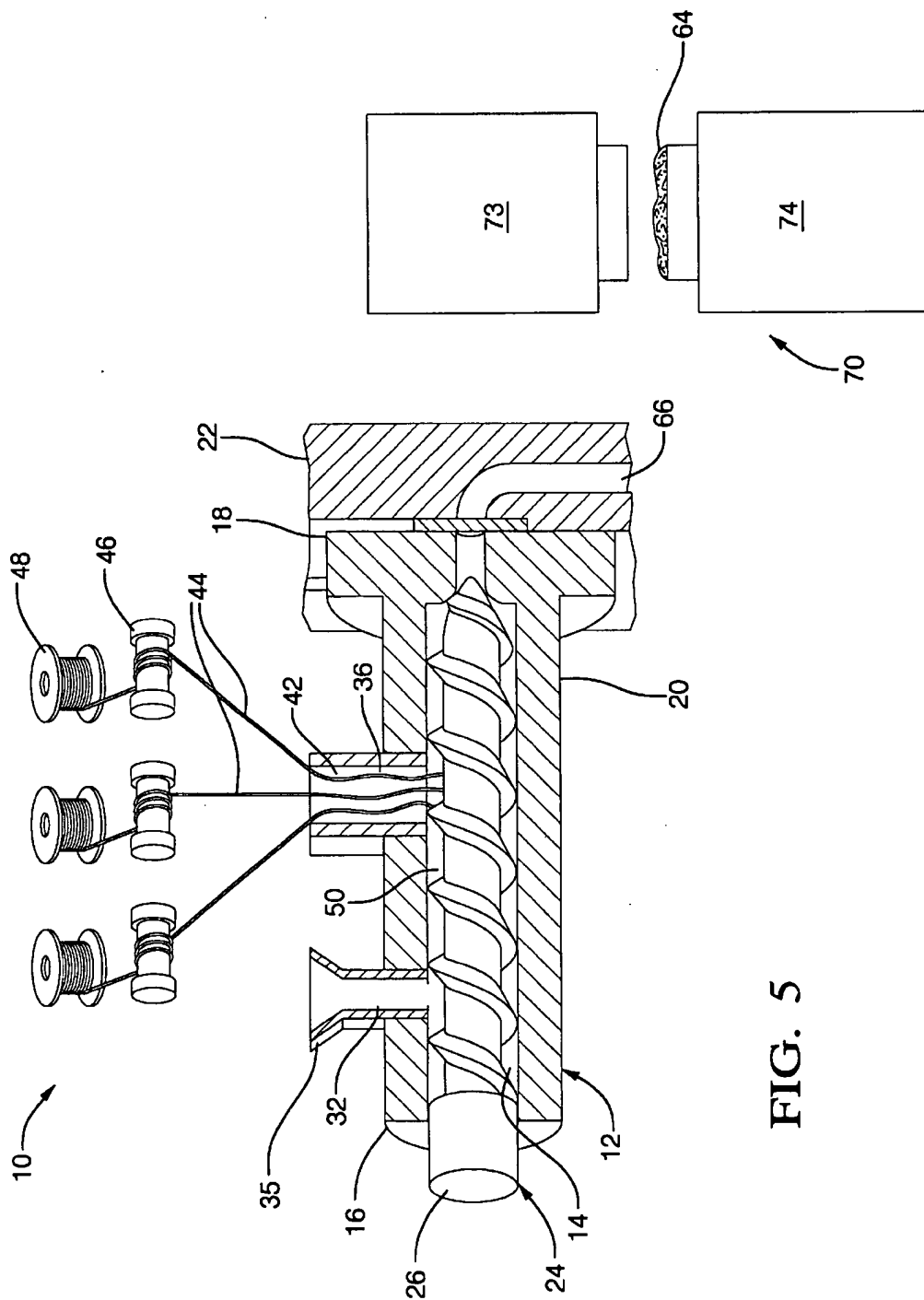


FIG. 5

1

IN-LINE COMPOUNDING/EXTRUSION DEPOSITION AND MOLDING APPARATUS AND METHOD OF USING THE SAME

TECHNICAL FIELD

The present invention relates generally to an apparatus and method of manufacturing a resin structure reinforced with long fibers and, more particularly, to an apparatus and method of manufacturing for a single-step in-line compounding of a reinforcing fiber with extrusion compression molding.

BACKGROUND OF THE INVENTION

Elongated resin structures reinforced with fibers in which thermoplastic resins are reinforced with continuous fibers have mechanical properties superior to those structures reinforced with short fibers. Such structures are beneficial because they can be cut and formed into pellets or similar materials. Elongated thermoplastic resin structures reinforced with fibers are generally manufactured by the so-called pultrusion method by impregnating a thermoplastic resin into a continuous reinforcement fiber bundle while the bundle is passed through a cross-head extrusion die, after which the resin-impregnated fiber bundle is drawn out through a die. After undergoing the pultrusion method, the structures are cut to a desired size.

Other processes are used to produce elongated thermoplastic resin structures reinforced with fiber, for example, where first the plastic is melted in a long single screw extruder which is fed to another single screw extruder. Next chopped strands are fed into the melt, and the reinforcing fiber melt is pumped into an accumulator after which the required log size is cut and fed into a vertical molding press.

Currently, there is no apparatus available employing a single-step process that utilizes a single screw extruder as a reinforcing fiber compounder and melt deposition unit. Likewise, there is no method currently available where reinforcing fibers are fed into a barrel of the apparatus such that the fibers are constantly maintained in a stretched condition regardless of the movement of the apparatus, thereby eliminating the possibility of fiber entanglement.

SUMMARY OF THE INVENTION

It is, therefore desirable to provide an apparatus and process for in-line compounding of reinforcing fiber and molding in a single-step process. A reinforcing product is compounded by use of a reciprocating single screw extruder having a reinforcing fiber compounder and a melt depositing unit, where the reinforcing fibers are severed at a maximum desirable length and kept in a stretched tensioned condition regardless of the apparatus positioning such that there are no loose or sagging fibers during the process.

Advantageously, the in-line compounding/extrusion apparatus of the present invention allows for the in-line compounding of reinforcing fibers with extrusion compression molding in a single step by utilizing a reciprocating single screw injection unit. Typically, the in-line compounding of reinforcing fibers with extrusion compression molding would involve high cost, bulky equipment consisting of combinations of single screw, twin screw and plunger deposition assemblies. By using a single-step process for the in-line compounding of reinforcing fibers and extrusion compression molding, the present invention offers a more cost effective method of producing a higher quality part.

In accordance with the preferred embodiment of the present invention, there is provided a method to incorporate

2

the continuous reinforcing fiber into the reciprocating single screw injection unit and to sever and uniformly impregnate the fine filaments with resin keeping maximum fiber length in the part.

In accordance with another aspect of the preferred embodiment of the present invention the apparatus includes winding/unwinding reels and guides which prevent the reinforcing fibers from sagging and breaking during a melt deposition step or during forward and rear movement of the reciprocating single screw injection unit.

The above and other objects and advantages of the invention will be apparent from the following description, the accompanying drawings and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Referring now to the drawings wherein like elements are numbered alike in the several Figures:

FIG. 1 is a cross-sectional side elevational view of one embodiment of an apparatus of the present invention in a first position;

FIG. 2 is a cross-sectional side view of the apparatus of FIG. 1 in a second position;

FIG. 3 is a cross-sectional side elevational view of the apparatus of FIG. 1 in a third position;

FIG. 4 is a cross-sectional side elevational view of the apparatus of FIG. 1 in a fourth position; and

FIG. 5 is a cross-sectional side elevational view of the apparatus of FIG. 1 in a fifth position.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

A typical embodiment of an in-line compounding/extrusion apparatus 10 embodying the present invention is shown in FIGS. 1-5. The apparatus 10 has a barrel 12 which in a preferred embodiment is cylindrical in shape. It will be appreciated that barrel 12 may have other shapes. Barrel 12 includes an internal cavity 14 formed therein and extending along a longitudinal axis thereof. The barrel 12 has a first end 16, an opposite second end 18 and an outer surface 20. The internal cavity 14 extends from the first end 16 to the second end 18 of the barrel 12. Located at the second end 18 of the barrel 12 is a die 22 including a blade 23 that opens and closes during operation of the apparatus 10 wherein the blade 23 is initially closed. The internal cavity 14 is preferably cylindrical in shape and has a diameter great enough to permit a screw 24 to be disposed therein. In the preferred embodiment, the screw 24 is sized to fit tightly in the internal cavity 14. In other words, the widest diameter of the screw 24 is slightly less than the inner diameter of the internal cavity 14. The barrel 12 preferably includes a beveled portion 19 which comprises an annular beveled surface which has a complementary shape as the head 30 so that the beveled portion 19 acts as a stop for the screw 24 as the screw 24 is driven in a direction towards the second end 18. Beveled portion 19 includes and defines a central opening 31.

The screw 24 includes a first end 26 and an opposing second end 28. A head 30 is provided at the second end 28 of the screw 24. When the screw 24 is inserted into the internal cavity 14 of the barrel 12, the head 30 is inserted into the internal cavity 14 at the first end 16 of the barrel 12 and is advanced therein towards the opposing second end 18. The apparatus 10 further has a die 22 that is in selective fluid communication with the internal cavity 14 at the second end 18 of the barrel 12. The blade 23 is designed to provide the

selective fluid communication between the internal cavity 14 and the die 22 so that in the closed position shown in FIGS. 1 and 2, the screw 24 is prevented from advancing material into the die 22. As a result, material disposed within the internal cavity 14 of the barrel 12 is prevented from freely entering or communicating with the die 22. As is known in the extruding art, the screw 24 has a plurality of flights 25 which are designed to advance the material through the internal cavity 14 as the screw 24 rotates and the material is picked-up and advanced forward by the plurality of flights 25.

Apparatus 10 also includes a first inlet 32 which is in communication with the barrel 12 and more specifically with the internal cavity 14. The exemplary first inlet 32 comprises a bore extending from the outer surface 20 of the barrel 12 and is generally perpendicular to the longitudinal axis of the barrel 12. The first inlet 32 opens into the internal cavity 14 so that an inlet material 34 may be introduced thereto from outside of the apparatus 10. In the exemplary illustrated embodiment, the first inlet 32 is preferably cylindrical in shape. It being understood that the first inlet 32 may have other cross-sectional shapes. The diameter of the first inlet 32 is of a sufficient dimension to permit the inlet material 34 to be introduced therethrough into the internal cavity 14. As the inlet material 34 enters the internal cavity 14, the screw 24 is designed to have the greatest flight depth so as to assure easy entry of the inlet material 34 and its conveyance forward under a high positive pressure. As the inlet material 34 is introduced into the internal cavity 14, it contacts the screw 24 and is disposed therearound and between the plurality of flights 25 which serves to advance the inlet material 34 once the screw 24 is rotated.

The apparatus 10 further includes a second inlet 36. Similar to the first inlet 32, the second inlet 36 comprises a bore extending from the outer surface 20 of the barrel 12. The second inlet 36 is generally perpendicular to the longitudinal axis of the barrel 12. The second inlet 36 opens into the internal cavity 14 so that material may be introduced thereto from outside of the apparatus 10. In the exemplary illustrated embodiment, the second inlet 36 is preferably cylindrical in shape. The second inlet 36 is positioned intermediate the first inlet 34 and the second end 18 of the barrel 12 and each respective axis of the first and second inlets 34, 36 are substantially parallel to one another. In the preferred embodiment, the second inlet 36 is cylindrical in shape; however, as can be appreciated, other shapes can be utilized. The first inlet 32 is thus closer to the first end 16 of the barrel 12 than the second inlet 36.

The first inlet 32 is designed to fit a hopper 35. The hopper 35 comprises a funnel-like holder capable of holding the inlet material 34. The inlet material 34 being fed into the internal cavity 14 through the first inlet 32 includes but is not limited to suitable thermoplastics and thermoset compounds. In one exemplary embodiment, the inlet material 34 comprises a quantity of plastic pellets which are fed through the hopper 35 and first inlet 32 into the internal cavity 14. The inlet material 34 is melted prior to further processing, wherein the melting is accomplished by maintaining a predetermined compression ratio of the screw 24 as the inlet material 34, e.g., plastic pellets, is advanced forward within the internal cavity 14 by the plurality of flights 25. As is known in the art, another method of describing the screw 24 is in terms of compression ratio. The compression ratio is generally defined as a comparison of the channel depth in the first flight of the first zone (feeding zone) and the channel depth of the last flight in the first zone (feeding zone). The channel depth (flight depth) is the distance from the outer

edge of a flight 25 to the outer surface of the screw 24. The screw 24 is designed so that as the inlet material 34 enters the internal cavity 14 through the first inlet 32, the depth of the plurality of flights 25 of the screw 24 is decreased. By decreasing the depth of the plurality of flights 25, the compression ratio is increased. Between the first and second inlets 32, 36 the depth of flights 25 transition quickly from a deep flight depth to a shallow flight depth. The flight depth should be deep enough to create a compression ratio greater than about 3.5:1 in the first zone. In an exemplary embodiment, the compression ratio is preferably about 8:1 to aid in the rapid melting of the incoming inlet material 34 before it reaches the next second inlet 36.

The second inlet 36 preferably includes a reinforcing fiber guide 42. The guide 42 comprises any number of guides which are designed to separate individual reinforcing fiber bundles 44 from one another so that the individual reinforcing fiber bundles 44 do not become entangled with one another as they are fed into the internal cavity 14. It being understood that the reinforcing fibers are commonly provided in reinforcing fiber bundles 44 which are then fed into the apparatus 10. An individual reinforcing fiber bundle 44 is also commonly referred to as a roving which comprises of a number of fibers with defined diameters and special sizing. For example, the second inlet 36 may be formed so that the guide 42 comprises at least one bore formed in and extending through the second inlet 36, wherein one reinforcing fiber bundle 44 is received within one bore. Each reinforcing fiber bundle 44 is formed of any suitable number of reinforcing fibers including but not limited to glass fibers, natural fibers, polyaramid fibers (e.g., Kevlar fibers commercially available from DuPont), carbon fibers or the like. Each reinforcing fiber bundle 44 is fed into the guide 42 from at least one winding/unwinding reel 46. The preferred embodiment is shown with three (3) winding/unwinding reels 46 for three (3) reinforcing fiber bundles 44; however as few as one (1) and as many as desired can be utilized in the present invention. The guide 42 is useful in directing the reinforcing fiber bundles 44 into the proper location in the internal cavity 14 and works in conjunction with the winding/unwinding reels 46 to keep the reinforcing fiber bundle 44 in a constant taut state. In the exemplary embodiment shown, the guide 42 comprises a rotatable member having a plurality of grooves formed therein for separating individual reinforcing fiber bundles 44 from one another so that the individual fiber bundles 44 do not become entangled during the feeding process.

The winding/unwinding reels 46 are preferably located above the guide 42 and are fed from an equal number of spools 48 containing the reinforcing fiber bundles 44. While the exemplary embodiment shows the spools 48 as having a round shape, it is understood that the other shapes may be used. There is one spool 48 feeding each winding/unwinding reel 46. The movement of the apparatus 10 and the screw 24 sets in motion the winding/unwinding of the reinforcing fiber bundles 44 on the reels 46. When the reinforcing fiber bundles 44 are unwound from the reels 46, the rotation of the screw 24 results in the feeding of the reinforcing fiber bundles 44 into the guide 42 and thus into the internal cavity 14 of the barrel 12 as well as the simultaneous plastification of the inlet material 34 which is introduced through the first inlet 32. The unwinding of the reinforcing fiber bundles 44 from the winding/unwinding reels 46 results in the accompanying unwinding from the associated spools 48. When the process is reversed, the reels 46 are wound as are the connected spools 48 so as not to allow for any slack in the reinforcing fiber bundles 44. Thus, the reinforcing fiber

5

bundles 44 are consistently under tension regardless of the positioning of the apparatus 10.

The internal cavity 14 of the barrel 12 is precisely sized to fit the screw 24 and allow a very narrow gap 50 to exist between the outer diameter of the screw 24 and the diameter of the internal cavity 14 of the barrel 12. The screw 24 has a preselected diameter (D) and length (L) such that L/D is large up to 35:1. Preferably the L/D ratio could be in the range of 20:1 to 35:1. It is generally known that the higher the L/D ratio, the higher will be the surface available for shearing, mixing, and plasticating the inlet material 34. Throughout operation the screw 24 will be free to rotate through the internal cavity 14, additionally the screw 24 is preset with back pressure allowing for the retraction (or movement away from the second end 18 of the barrel 12) of the screw 24 once an accumulation of material forms in front of the screw 24. The front of the screw 24 is defined as that area between the head 30 of the screw 24 and the blade 23 when it is in a closed position. This is known as "shot size".

The length of the barrel 12 is generally divided into at least three (3) zones, namely a first zone 52, a second zone 56, and a third zone 62. Each of the zones 52, 56, 62 performs an operation useful in the compounding/extrusion process as will be described in greater detail hereinafter. The first zone or a melting zone 52 is created as the inlet material 34 is fed into the first inlet 32 under compression to form a melted plastic. The screw 24 design in this area has a deep flight depth to create a high compression ratio so that the inlet material 34 rapidly melts as the inlet material 34 is introduced and advanced through the first zone 52. However, the flight depth in the first zone 52 reduces sharply from the beginning to the end of the zone. As the melted inlet material 34 leaves the first zone 52, it enters a second zone or a preparation and cutting zone 56. Thus, the first zone 52 serves to melt the inlet material 34 for further processing in the apparatus 10. It being understood that the relative size of each of the zones 52, 56, and 62 has been illustrated for purpose of illustration and clarity only and it is within the scope of the present invention that the lengths of these zones 52, 56, and 62 differ depending upon the application as is known in the art.

The second zone 56 is established to prepare the reinforcing fiber bundles 44 for shearing and impregnation as they are introduced into the apparatus 10 and more specifically the internal cavity 14. The screw 24 design in this second zone 56 has a deep flight depth however the flight depth throughout the second zone 56 remains constant so that there is zero compression in the second zone 56. In other words, the compression ratio is zero because there is no change in the depth of the flights 25 in the second zone 54. The actual screw flight depth depends on the number of reinforcing fiber bundles 44 fed into the second zone 56 and the type of the inlet material 34 used. A deep flight depth is necessary in the second zone 56 so as to accommodate a larger volume of reinforcing fiber bundles 44. The flight depth should be as high as possible based on the structural integrity of the screw 24 which is dependant on the screw diameter. As the reinforcing fiber bundles 44 pass from the guide 42 into the internal cavity 14, the filaments of each of the reinforcing fiber bundles 44 are opened for proper melt impregnation. That is the filaments of the reinforcing fiber bundles 44 are opened for better wetting so that each filament can be coated with the melted inlet material 34. It is also understood that the may pre-heat the reinforcing fiber bundles 44 after the reinforcing fiber bundles 44 pass the winding/unwinding reels 46 but prior to entrance into the second inlet 36. This results in increased wettability of the individual reinforcing fiber bundles 44 with the melted inlet material 34.

6

In the second zone 56, the reinforcing fiber bundles 44 are sheared or broken to a desirable longer length. The shearing is accomplished as the tensile load on the reinforcing fiber bundles 44 is increased so that each of the reinforcing fiber bundles 44 shears in approximately the same length. As the reinforcing fiber bundles 44 move through the second zone 56, the resistance on the reinforcing fiber bundles 44 increases so that when the resistance becomes too great, the reinforcing fiber bundles 44 are sheared or broken forming individual sheared reinforcing fibers 60.

Upon exiting the second zone 56, the sheared reinforcing fibers 60 exit along with the melted inlet material 34 and the further mixing begins. The third zone 62 comprises a mixing and impregnation zone for further mixing of the melted inlet material 34 and the sheared reinforcing fibers 60. The continued mixing and impregnation result in a fiber filled melt being produced. The fiber filled melt is generally indicated at 64 in the Figures.

The apparatus 10 further includes an outlet 66 formed in the die 22 which serves as an exit for the fiber filled melt 64 from the die 22 after the fiber filled melt 64 travels from the barrel 12 to the die 22. The outlet 66 preferably extends from the die 22 in a direction perpendicular to the longitudinal axis of the die 22 and continues through the die 22 before reaching an outer surface of the die 22. In the preferred embodiment, the outlet 66 is cylindrical in shape however other shapes can be utilized, e.g., such as ribbons or sheet shapes. The outlet 66 is located near the second end 18 of the barrel 12 and at least a portion thereof is preferably generally parallel to the first and second inlets 32, 36, however the outlet 66 extends in a direction opposite the first and second inlets 32, 36.

As shown in FIGS. 1-5, a method of using the in-line compounding/extrusion deposition compression molding apparatus 10 will now be described in greater detail. The present invention provides a process for preparing the moldable fiber filled melt 64. The fiber filled melt 64 is produced from the mixing of the melted inlet material 34 and the sheared reinforcing fibers 60. Molded structures that are reinforced with long reinforcing fibers have mechanical properties superior to those structures reinforced with short fibers. To enjoy the benefits of superior mechanical properties, the process of this invention allows for a long reinforcing fiber to be maintained without breakage and therefore cut at a longer length than was previously possible in a single step process. In the process, the apparatus 10 of the present invention mixes the longer cut reinforcing fiber bundles 44 with the melted inlet material 34 before deposition in a tool 70. As shown in FIG. 1, the apparatus 10 and more specifically the barrel 12 and the die 22 are in a first position relative to the winding/unwinding reels 46 and the tool 70. In the first position, the die 22 is not axially aligned with the tool 70 but rather the barrel 12 and the die 22 are off set therefrom.

In the exemplary and illustrated embodiment in the first position, the second inlet 36 is generally axially aligned with a centermost winding/unwinding reel 46. In the first position, the screw 24 generally is disposed within the barrel 12 so that the head 30 is adjacent to or abuts the complementary beveled portion 19 of the barrel 12. The beveled portion 19 comprises an annular beveled surface which has a complementary shape as the head 30 so that the beveled portion 19 acts as a stop for the screw 24. It being understood that the illustrated embodiment shown in the Figures is merely exemplary in nature and the present invention is not limited to the illustrated embodiment. In the first position, the apparatus 10 is stationary and the reinforcing

fiber bundles 44 are disposed within the winding/unwinding reel 46 and extend through the second inlet 36 into the internal cavity 14 so that the reinforcing fiber bundles 44 contact the screw 24 before operation of the apparatus 10.

As shown in FIG. 2, the process begins by rotating the screw 24 located inside the internal cavity 14 of the barrel 12. Preferably, a beginning rotation speed would be dependant on the type of inlet material 34 and the amount of the cut reinforcing fibers 60 in the final compound. Not shown is the means by which the rotation of screw 24 is accomplished. Any conventional means for rotation can be utilized. Simultaneously, the inlet material 34 is fed under compression into the internal cavity 14 of the barrel 12. The melting of the inlet material 34 is achieved in the first zone 52 of the apparatus 10. The first zone 52 is where the first operation of the apparatus is preformed. Preferably the inlet material 34 is fed under a high compression ratio, up to about 8:1 for the rapid melting of the inlet material 34. The flight depth in the first zone 52 rapidly transitions from a deep flight depth to a shallow flight depth within the first zone 52.

As the inlet material 34 is melted, the screw 24 rotation continuously feeds the reinforcing fiber bundles 44 into the second zone 56. The reinforcing fiber bundles 44 are fed into the second zone 56 through the guide 42 from the series of winding/unwinding reels 46 which work in connection with the movement of the apparatus 10 to keep the reinforcing fiber bundles 44 in a constant taut state. The screw 24 preferably has a free flowing check valve to prevent unintentional reinforcing fiber bundles 44 breakage. The reinforcing fiber bundles 44 are thus introduced into the apparatus 10 after the inlet material 34 has been melted in the first zone 52 due to the rotation of the screw 24. Thus, the plastication process begins prior to the introduction of the reinforcing fiber bundles 44 into the internal cavity 14 so that melted inlet material 34 is advanced into the second zone 56 for combination with the reinforcing fiber bundles 44. Rotation of the winding/unwinding reels 46 is controlled based on the apparatus 10 location during operation. The movement of the winding/unwinding reels 46 can be generated by a servo-driven motor with closed loop control or by pretension created by spring loading the reel 46 or any other known mechanical means. When closed loop control is chosen as the mechanism, the servo-driven motor either unwinds or winds the reinforcing fiber bundles 44 depending upon the relative position of the apparatus 10. Prior to moving into the second zone 56, the reinforcing fiber bundles 44 can be preheated to a temperature at or above the plastic melt temperature of the inlet material 34 to improve melt mixing and homogenization that takes place in the third zone 62. A higher melt temperature allows for better wetting of the reinforcing fiber bundles 44 by the melted inlet material 34.

The rotation of the screw 24 continuously feeds the reinforcing fiber bundles 44 into the internal cavity 14 at the second zone 56 and as the reinforcing fiber bundles 44 enter the second zone 56, the reinforcing fiber bundles 44 are unwound from the winding/unwinding reels 46 keeping the reinforcing fiber bundles 44 stretched. Once in the second zone 56, the filaments of the reinforcing fiber bundles 44 are opened for improved wetting and the stretched reinforcing fiber bundles 44 are sheared or broken to a desirable longer length. Shearing is achieved, for example, by increasing the tensile load on the reinforcing fiber bundles 44 and when the resistance becomes too great, the reinforcing fiber bundles 44 shear forming the sheared reinforcing fibers 60. As the inlet material 34 is melted and the reinforcing fiber bundles 44 sheared, the apparatus 10 is stationary, as best shown in FIG. 2.

As the reinforcing fiber bundles 44 are sheared in the second zone 56, the melted inlet material 34 begins to mix with the cut reinforcing fibers 60 to create a fiber filled melt 64. Continued mixing occurs as the fiber filled melt 64 is advanced into the third zone 62 by the rotation of the plurality of flights 25. The third zone 62 accomplishes the uniform distributive mixing and impregnation of the sheared reinforcing fibers 60 with the melted inlet material 34 to form the fiber filled melt 64. As the fiber filled melt 64 accumulates, it is advanced into an accumulation zone, generally indicated at 68 because the plurality of flights 25 continues to advance the fiber filled melt 64. The accumulation zone 68 is that area between the head 30 of the screw 24 and the blade 23. As can be appreciated the accumulation zone 68 increases as the screw 24 retracts in a direction away from the blade 23. The screw 24 retracts based on its back pressure setting and the retraction occurs as more and more fiber filled melt 64 builds up in the accumulation zone 68. Accumulation of the fiber filled melt 64 occurs until enough is gathered to create a shot size having a predetermined size. Because the blade 23 is in a closed position, the fiber filled melt 64 continues to build between the head 30 and the blade 23 and this build-up causes the back pressure which drives the screw 24 in a direction away from the blade 23. As shown in FIG. 2, the screw 24 assumes a second retracted position in which the screw 24 has been driven in a direction away from the blade 23 to accommodate the fiber filled melt 64 between the head 30 and the blade 23.

As shown in FIG. 3, once the proper shot size is detected, the apparatus 10 moves in a direction towards the tool 70. An exemplary tool 70 comprises a press 73 with a mold 74. As mentioned above the apparatus 10 is moved by known means. Because the winding/unwinding reels 46 and spools 48 are preferably stationary relative to the barrel 12 and die 22, the movement of the barrel 12 and die 22 in the direction towards the tool 70 causes the winding/unwinding reels 46 to unwind to release an appropriate length of each of the reinforcing fiber bundles 44 to accommodate the movement of the barrel 12 and the die 22. This results because even in the stage shown in FIG. 3, the reinforcing fiber bundles 44 are fed into the second inlet 36 and communicate with the internal cavity 14 and the screw 24 disposed therein so that ends of the reinforcing fiber bundles 44 are not free but rather are secured within the internal cavity 14 so that it is taut (under tension). Thus, the movement of the barrel 12 and the die 22 towards the tool 70 results in the reinforcing fiber bundles 44 being angled relative to the barrel 12 so that the reinforcing fiber bundles 44 feed through the guide 42 and into the second inlet 36 as shown in FIG. 3.

Once the apparatus 10 reaches an edge 71 of the tool 70, the blade 23 in the die 22 opens and the screw 24 moves forward forcing the fiber filled melt 64 out through the outlet 66 and deposits the fiber filled melt 64 as the apparatus 10 moves as programmed to distribute the fiber filled melt 64 over the mold 74. Because the apparatus 10 is capable of moving in three dimensions X, Y, and Z, the apparatus 10 is capable of distributing the fiber filled melt 64 by moving in the programmed X, Y, and Z directions to evenly distribute the fiber filled melt 64 in the mold 74. Because movement of the barrel 12 or screw 24 effects the existing tension of the reinforcing fiber bundles 44, the winding/unwinding reels 46 are designed to either wind or unwind the reinforcing fiber bundles 44 so that sagging and breakage of the reinforcing fiber bundles 44 are prevented. As shown in FIG. 3, the winding/unwinding reels 46 unwind the reinforcing fiber bundles 44 with constant tension to permit movement of the barrel 12 and die 22 towards the tool 70. In this deposition

9

stage, the screw 24 is driven towards the beveled surface 19 to once again assume the first position and cause the fiber filled melt 64 to be displaced through the central opening 31 and into the outlet 66 of the die 22 where the fiber filled melt 64 is then directed into the mold 74.

Referring now to FIGS. 4 and 5, FIG. 4 illustrates the apparatus 10 as the fiber filled melt 64 is deposited onto the mold 74. Once the apparatus 10 is properly positioned, the blade 23 is opened so that the fiber filled melt 64 may be deposited onto the mold 74. After the fiber filled melt 64 is deposited onto the mold 74, the press 73 is driven so as to close the tool 70 and compress, thereby forming the desired part by a compression molding technique. As shown in FIG. 5, after the fiber filled melt 64 is deposited and prior to the press 73 being driven towards the mold 74, the barrel 12 and the die 22 move back to the first position illustrated generally in FIG. 1. At this time, the blade 23 is repositioned to the closed position so that the process may be repeated. When the barrel 12 and the die 22 move back the first position, the reinforcing fiber bundles 44 are wound up by the winding/unwinding reels 46 so as to take up the potential slack which would be created by returning the barrel 12 and die 22 to the original first position. Because the winding/unwinding reels 46 are preferably spring loaded, the reinforcing fiber bundles 44 are not permitted to sag but rather remain under constant tension as the barrel 12 and the die 22 move either in the direction towards the tool 70 or in the direction away from the tool 70. This movement of the barrel 12 and the die 22 also likewise permits the press 73 to be driven towards and contact the mold 74 to produce the compressed formed part. Once the formed part cools, the tool 70 is opened and the molded fiber filled part is removed.

The present invention advantageously provides apparatus 10 and process for in-line compounding of reinforcing fiber bundles 44 and molding 34 in a single step process. The exemplary apparatus 10 compounds a reinforcing product by use of the reciprocating single screw 24 having a reinforcing fiber compounder and melt depositing unit. According to the present invention, the reinforcing fiber bundles 44 are severed at a maximum desirable length and are maintained in a stretched tensioned condition regardless of the positioning of apparatus 10 such that the reinforcing fiber bundles 44 are not loose and does not sag during the process. By using a single-step process for the in-line compounding of reinforcing fibers and extrusion compression molding, the present invention offers a more cost effective method of producing a higher quality part because a single apparatus is used instead of the multiple part assemblies used conventionally. Additionally, the present invention incorporates the reinforcing fiber bundles 44 into the reciprocating single screw injection unit and severs and uniformly impregnates the fine filaments with resin keeping maximum fiber length in the manufactured product. Due to the longer reinforcing fiber retention in the manufactured product, a higher strength product can be produced.

It will be understood that a person skilled in the art may make modifications to the preferred embodiment shown herein within the scope and intent of the claims. While the present invention has been described as carried out in a specific embodiment thereof, it is not intended to be limited thereby but is intended to cover the invention broadly within the scope and spirit of the claims.

What is claimed is:

1. A process for in-line compounding of a reinforcing fiber bundle with extrusion compression molding using an in-line compounding/extrusion deposition and molding apparatus, the process comprising:

10

providing a first material to a first inlet formed in the apparatus, the first inlet being in fluid communication with an internal cavity of the apparatus, the internal cavity having a single rotatable extruder screw, the first material being fed into a first zone of the internal cavity;

providing at least one reinforcing fiber bundle to a second inlet formed in the apparatus, the at least one reinforcing fiber bundle being fed into a second zone of the internal cavity under a predetermined constant tension to prevent slack in the at least one reinforcing fiber bundle;

rotating the single rotatable extruder screw so as to melt the first material in the first zone prior to advancing the melted first material to the second zone, the rotation of the single rotatable extruder screw causing the at least one reinforcing fiber bundle to be fed into the second zone under the predetermined constant tension;

shearing the at least one reinforcing fiber bundle in the second zone;

mixing the sheared at least one reinforcing fiber bundle and the melted first material in a third zone to produce a fiber bundle filled melt;

retracting the single rotatable extruder screw to permit expansion of a fourth zone, wherein the fiber filled melt accumulates in the fourth zone thereby forming a shot; and

directing the fiber bundle filled melt through an outlet formed in the apparatus by movement of the single rotatable extruder screw in a direction toward the outlet.

2. The process according to claim 1, wherein the first material comprises a material selected from the group consisting of thermoplastic materials and thermoset materials.

3. The process according to claim 1, wherein providing at least one reinforcing fiber bundle to the second inlet comprises:

unwinding the at least one reinforcing fiber bundle from a spool;

and maintaining the predetermined tension by passing the at least one reinforcing fiber bundle over a winding/unwinding reel so that the at least one reinforcing fiber bundle is under constant tension as the at least one reinforcing fiber bundle is fed into the second zone.

4. The process according to claim 3, wherein the winding/unwinding reel is spring loaded so that slack in the at least one reinforcing fiber bundle is prevented during operation of the apparatus and the tensile load on the at least one reinforcing fiber bundle is maintained below the predetermined value.

5. The process according to claim 1, wherein shearing the at least one reinforcing fiber bundle comprises placing a tensile load on the at least reinforcing fiber bundle so that the tensile load exceeds a predetermined value and causes the at least one reinforcing fiber bundle to shear.

6. The process according to claim 1, wherein the single rotatable extruder screw has a first compression ratio in the first zone to cause the inlet material to rapidly melt in the first zone prior to the melted first material being advanced into the second zone.

7. The process according to claim 6, wherein the first compression ratio is from about 3.5:1 to about 8:1.

8. The process according to claim 1, wherein the second zone includes a second compression ratio, wherein the second compression ratio is constant in the second zone.

11

9. The process according to claim 1, further including:
moving the apparatus to a predetermined mold position
prior to directing the fiber bundle filled melt from the
outlet.

10. The process according to claim 9, wherein moving the
apparatus comprises moving the apparatus in at least one
direction of a three dimensional area.

11. A process for in-line manufacturing of a fiber rein-
forced molded structure, the process comprising:

feeding a first material into a first inlet of an apparatus,
said first inlet being in fluid communication with an
internal cavity of said apparatus;

advancing said first material from said first inlet to a
second inlet of said apparatus by rotating a single
extruder screw within said internal cavity such that said
first material is melted, said second inlet being in fluid
communication with said internal cavity;

feeding reinforcing fiber under a predetermined tension
into said second inlet by rotating said single extruder
screw within said internal cavity such that a fiber filled
melt is formed from said first material and said rein-
forcing fiber;

advancing said fiber filled melt from said second inlet to
an outlet of said internal cavity by rotating said single
extruder screw within said internal cavity;

accumulating a shot of said fiber filled melt at said outlet
by moving said single extruder screw from a first
position to a second position;

distributing said shot on a mold by opening said outlet,
moving said single extruder screw from said second

12

position to said first position, and moving said outlet
with respect to said mold; and

closing said mold to form the fiber reinforced molded
structure from said shot.

12. The process as in claim 11, wherein said inlet material
comprises material selected from the group consisting of
thermoplastic materials and thermoset materials.

13. The process as in claim 12, wherein said reinforcing
is selected from the group consisting of glass fibers, natural
fibers, polyaramid fibers, and carbon fibers.

14. The process as in claim 11, wherein moving said outlet
with respect to said mold further comprises:

moving said apparatus in at least one direction of a three
dimensional area.

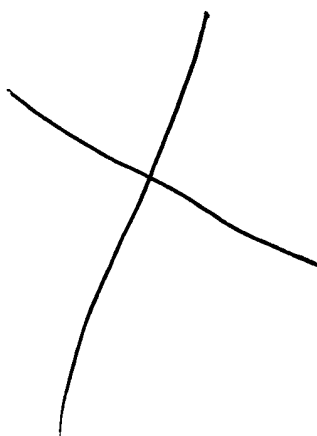
15. The process as in claim 11, wherein feeding said
reinforcing fiber under said predetermined tension com-
prises:

unwinding said reinforcing fiber from a spool; and

maintaining said predetermined tension by passing said
reinforcing fiber over a winding/unwinding reel so that
said reinforcing fiber is under said predetermined ten-
sion as said reinforcing fiber is fed into said second
inlet.

16. The process as in claim 15, wherein said predeter-
mined tension prevents sagging and premature shearing of
said reinforcing fiber.

* * * * *



This Page Blank (uspto)



US006403784B1

(12) **United States Patent**
Turner, Jr. et al.(10) **Patent No.:** **US 6,403,784 B1**
(45) **Date of Patent:** **Jun. 11, 2002**(54) **HUMAN UNCOUPLING PROTEINS AND
POLYNUCLEOTIDES ENCODING THE
SAME**(75) **Inventors:** **C. Alexander Turner, Jr.; Brian
Mathur; Brian Zambrowicz; Arthur
T. Sands, all of The Woodlands, TX
(US)**(73) **Assignee:** **Lexicon Genetics Incorporated, The
Woodlands, TX (US)**(*) **Notice:** Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.(21) **Appl. No.:** **09/501,558**(22) **Filed:** **Feb. 9, 2000****Related U.S. Application Data**(60) **Provisional application No. 60/158,458, filed on Oct. 8,
1999, and provisional application No. 60/119,228, filed on
Feb. 9, 1999.**(51) **Int. Cl.⁷** **C07H 21/04; C07H 21/02;
C07K 14/00; C07K 1/00**(52) **U.S. Cl.** **536/23.5; 536/23.1; 530/350;
530/300**(58) **Field of Search** **536/23.1, 23.5;
530/300, 350, 388.1; 435/6; 514/12**(56) **References Cited****U.S. PATENT DOCUMENTS**

4,190,496 A	2/1980	Rubenstein et al.
4,215,051 A	7/1980	Schroeder et al.
4,376,110 A	3/1983	David et al.
4,683,202 A	7/1987	Mullis
4,873,191 A	10/1989	Wagner et al.
4,946,778 A	8/1990	Ladner et al.
5,075,217 A	12/1991	Weber
5,364,759 A	11/1994	Caskey et al.
5,399,346 A	3/1995	Anderson et al.
5,625,048 A	4/1997	Tsien et al.
5,702,902 A	12/1997	Tartaglia
5,741,666 A	4/1998	Tartaglia
5,780,280 A	7/1998	Lebkowski et al.
5,824,544 A	10/1998	Armentano et al.
5,827,703 A	10/1998	Debs et al.
5,830,727 A	11/1998	Wang et al.
5,837,458 A	11/1998	Minshull et al.
5,840,710 A	11/1998	Lee et al.
5,843,742 A	12/1998	Natsoulis et al.
5,846,528 A	12/1998	Podsakoff et al.
5,853,975 A	12/1998	Tartaglia
5,858,740 A	1/1999	Finer et al.

FOREIGN PATENT DOCUMENTS

WO	WO98/03243	1/1998
WO	WO 9831396	7/1998
WO	WO 9845313	10/1998
WO	WO 9845438	10/1998
WO	WO98/50542	11/1998

OTHER PUBLICATIONSSanchis et al., GenBank Accession No: 095258, Swis-
sProt-39 Database, Dec. 18, 1998.*Sanchis et al., GenBank Accession No: AF076981, Gen-
Embl Database, Jan. 10, 1999.*Sanchis et al., J. of Biological Chemistry, vol. 273, No. 51,
pp34611-34615, Dec. 18, 1998.*Sanchis et al., GenBank Accession No: 095258, Swis-
sProt-39 Database, May 30, 2000.*NCI-CGAP, GenBank Accession No: A1128486, EST
Database, Oct. 27, 1998.*Bird et al, 1988, "Single-Chain Antigen-Binding Proteins",
Science 242:423-426.Bitter et al, 1987, "Expression and Secretion Vectors for
Yeast", Methods in Enzymology 153:516-544.Butler, J.E., 1981, "The Amplified ELISA: Principles of and
Applications for the Comparative Quantitation of Class and
Subclass Antibodies and the Distribution of Antibodies and
Antigens in Biochemical Separates", Meth. Enzymol.
73:482-523.Colbere-Garapin et al, 1981, "A New Dominant Hybrid
Selective Marker for Higher Eukaryotic Cells", J. Mol. Biol.
150:1-14.Cote et al, 1983, "Generation of human monoclonal anti-
bodies reactive with cellular antigens", Proc. Natl. Acad.
Sci. USA 80:2026-2030.Gautier et al, 1987, "α-DNA IV:α-anomeric and β-ano-
meric tetrathymidylates covalently linked to intercalating
oxazopyridocarbazole. Synthesis, physiochemical proper-
ties and poly (rA) binding", Nucleic Acids Research
15(16):6625-6641.Gordon, 1989, "Transgenic Animals", Intl. Rev. Cytol.
115:171-229.Greenspan et al, 1993, "Idiotypes: structure and immuno-
genicity", FASEB Journal 7:437-444.Gu et al, 1994, "Deletion of a DNA Polymerase β Gene
Segment in T Cells Using Cell Type-Specific Gene Target-
ing", Science 265:103-106.Huse et al, 1989, "Generation of a Large Combinatorial
Library of the Immunoglobulin Repertoire in Phage
Lambda", Science 246:1275-1281.Huston et al, 1988, "Protein engineering of antibody binding
sites: Recovery of specific activity in an anti-digoxin sin-
gle-chain Fv analogue produced in *Escherichia coli*", Proc.
Natl. Acad. Sci. USA 85:5879-5883.Inoue et al, 1987, "Sequence-dependent hydrolysis of RNA
using modified oligonucleotide splints and R Nase H",
FEBS Letters 215(2):327-330.

(List continued on next page.)

Primary Examiner—Karen Cochrane Carlson**Assistant Examiner**—Rita Mitra(57) **ABSTRACT**The present invention relates to methods and compositions
for the treatment of biological disorders regulatable by the
controlled expression or inhibition of the described NUCPs.**5 Claims, No Drawings**

OTHER PUBLICATIONS

- Inoue et al, 1987, "Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides", *Nucleic Acids Research* 15(15):6131-6149.
- Inouye & Inouye, 1985, "Up-promoter mutations in the lpp gene of *Escherichia coli*", *Nucleic Acids Research* 13(9):3101-3110.
- Janknecht et al, 1991, "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus", *Proc. Natl. Acad. Sci. USA* 88:8972-8976.
- Kohler and Milstein, 1975, "Continuous cultures of fused cells secreting antibody of predefined specificity", *Nature* 256:495-497.
- Lakso et al, 1992, "Targeted oncogene activation by site-specific recombination in transgenic mice", *Proc. Natl. Acad. Sci. USA* 89:6232-6236.
- Lavitrano et al, 1989, "Sperm Cells as Vectors for Introducing Foreign DNA into Eggs: Genetic Transformation of Mice", *Cell* 57:717-723.
- Lo, 1983, "Transformation by Iontophoretic Microinjection of DNA: Multiple Integrations without Tandem Insertions", *Mol. & Cell. Biology* 3(10):1803-1814.
- Logan et al, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection", *Proc. Natl. Acad. Sci. USA* 81:3655-3659.
- Lowy et al, 1980, "Isolation of Transforming DNA: Cloning the Hamster apt Gene", *Cell* 22:817-823.
- Miyajima et al, 1986, "Expression of murine and human granulocyte-macrophage colony-stimulating factors in *S. cerevisiae*: mutagenesis of the potential glycosylation sites", *EMBO J.* 5:6, 1193-1197.
- Mulligan & Berg, 1981, "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase", *Proc. Natl. Acad. Sci. USA* 78(4):2072-2076.
- Neuberger et al, 1984, "Recombinant antibodies possessing novel effector functions", *Nature* 312:604-608.
- Nisonoff, 1991, "Idiotypes: Concepts and Applications", *J. of Immunology* 147:2429-2438.
- O'Hare et al, 1981, "Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase", *Proc. Natl. Acad. Sci. USA* 78(3):1527-1531.
- Platt, et al., 1994, "Independent Regulation of Adipose Tissue-specificity and Obesity Response of the Adipsin Promoter in Transgenic Mice", *J. Biol. Chem.* 269:28558-28562.
- Ruther et al, 1983, "Easy identification of cDNA clones", *EMBO Journal* 2(10):1791-1794.
- Santerre et al, 1984, "Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells", *Gene* 30:147-156.
- Sarin et al, 1988, "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphosphonates", *Proc. Natl. Acad. Sci. USA* 85:7448-7451.
- Smith et al, 1983, "Molecular Engineering of the Autographa californica Nuclear Polyhedrosis Virus Genome: Deletion Mutations within the Polyhedrin Gene", *J. Virol.* 46(2):584-593.
- Stein et al, 1988, "Physicochemical properties of phosphorothioate oligodeoxynucleotides", *Nucleic Acids Research* 16(8):3209-3221.
- Szybalska & Szybalski, 1962, "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait", *Proc. Natl. Acad. Sci. USA* 48:2026.
- Takeda et al, 1985, "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences", *Nature* 314:452-454.
- Thompson et al, 1989, "Germ Line Transmission and Expression of a Corrected HPRT Gene Produced by Gene Targeting in Embryonic Stem Cells", *Cell* 56:313-321.
- Van Der Putten et al, 1985, "Efficient insertion of genes into the mouse germ line via retroviral vectors", *Proc. Natl. Acad. Sci. USA* 82:6148-6152.
- Van Heeke & Schuster, 1989, "Expression of Human Asparagine Synthetase in *Escherichia coli*", *J. Biol. Chem.* 264:5503-5509.
- Voller, A. et al, 1978, "Enzyme immunoassays with special reference to ELISA techniques", *J. Clin. Pathol.* 31:507-520.
- Ward et al, 1989, "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", *Nature* 341:544-546.
- Wigler et al, 1977, "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells", *Cell* 11:223.
- Wigler et al, 1980, "Transformation of mammalian cells with an amplifiable dominant-acting gene", *Proc. Natl. Acad. Sci. USA* 77(6):3567-3570.
- Database EMBL Nucleotide And Protein Sequences, 1998, XP002141168 Hinxton, GB, Soares_pregnant_uterus_Nb-HPU *Homo sapiens* cDNA clone, NCI-CGAP; National Cancer Institute, Cancer Genome Anatomy Project, Tumor Gene Index <http://www.ncbi.nlm.nih.gov/ncicgap>.
- Database EMBL Nucleotide And Protein Sequences, 1998, XP002141171, Hinxton, GB, Soares_2NbMT Mus musculus cDNA clone Image:1265034, Mitochondrial Uncoupling Protein 2; mRNA sequence, Washington University School of Medicine.
- Database EMBL Nucleotide And Protein Sequences, 2000, XP002141172, Hinxton, GB, NCI_CGAP_Sub4 *Homo sapiens* cDNA clone, National Cancer Institute, Cancer Genome Anatomy Project (CGAP), Tumor Gene Index <http://www.ncbi.nlm.nih.gov/ncicgap>.
- Database EMBL Nucleotide And Protein Sequences, 2000, XP002141173, Hinxton, GB, Soares_NFL_T_GBC-S1 *Homo sapiens* cDNA clone, National Cancer Institute, Cancer Genome Anatomy Project (CGAP), Tumor Gene Index <http://www.ncbi.nlm.nih.gov/ncicgap>.
- Mao et al, 1998, "UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells", *FEBS Letters* 443(3):326-330.
- Sanchis et al, 1998, "BMCP1, a Novel Mitochondrial Carrier with High Expression in the Central Nervous System of Humans and Rodents, and Respiration Uncoupling Activity in Recombinant Yeast", *Journal of Biological Chemistry*, 273(51):34611-34615.

* cited by examiner

1

HUMAN UNCOUPLING PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims priority to U.S. Application Ser. No. 60/119,228, filed Feb. 9, 1999, and U.S. Application Ser. No. 60/158,458, filed Oct. 8, 1999, which are herein incorporated by reference in their entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotide sequences and the novel polypeptides encoded thereby. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins or polypeptides, and fusion proteins and peptides derived therefrom, antibodies to the encoded proteins or peptides, and genetically engineered animals that lack functional forms of the genes encoding the disclosed sequences, over express the disclosed sequences, as well as antagonists and agonists of the proteins, along with other compounds that modulate the expression or activity of the proteins encoded by the disclosed sequences that can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of physiological or behavioral disorders, or otherwise improving the quality of life.

2. BACKGROUND OF THE INVENTION

Uncoupling proteins (UCPs) are found in the mitochondria, but are encoded within the nucleus. In the mitochondria, UCPs uncouple, or regulate, the gradient that drives energy production in the cell/body. As such, UCPs effectively modulate the efficiency of energy production in the body, and hence body metabolism. Given the role of UCPs in the body, they are thought to be important targets for the study of thermogenesis, obesity, cachexia, and other metabolically related physiological functions, diseases, and disorders.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human UCPs, and the corresponding amino acid sequences encoded by the disclosed sequences. The novel human uncoupling proteins (NUCPs) described for the first time herein share structural relatedness with other mammalian uncoupling proteins and brain mitochondrial carrier proteins. The novel human nucleic acid sequences described herein encode proteins of 291 and 293 amino acids in length (see SEQ ID NOS:2 and 4).

A murine homologue of the described NUCPs has been identified and a "knockout" ES cell line has been produced using the methods described in U.S. application Ser. No. 08/942,806, herein incorporated by reference. Alternatively, such knockout cells and animals can be produced using conventional methods for generating genetically engineered animals and cells (see, for example, PCT Applic. No. PCT/US98/03243, filed Feb. 20, 1998, herein incorporated by reference). Accordingly, an additional aspect of the present invention includes knockout cells and animals having genetically engineered mutations in gene encoding the presently described NUCPs.

The invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, and the expression products of such nucleotides, and: (a)

2

nucleotides that encode mammalian homologs of the described genes, including the specifically described NUCPs, and the NUCP products; (b) nucleotides that encode one or more portions of the NUCPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NUCPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of NUCP, or one of its domains (e.g., a transmembrane domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide.

The invention also encompasses agonists and antagonists of NUCPs, including small molecules, large molecules, mutant NUCPs, or portions thereof that compete with or bind to native NUCPs, antibodies, and nucleotide sequences that can be used to inhibit the expression of the described NUCPs (e.g., antisense, ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NUCPs (e.g., expression constructs that place the described genes under the control of a strong promoter system), as well as transgenic animals that express a NUCP transgene, or "knockouts" (which can be conditional) that do not express functional NUCP.

Further, the present invention also relates to methods for using of the described NUCP products for the identification of compounds that modulate, i.e., act as agonists or antagonists, of NUCP expression and/or NUCP product activity. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptomatic representations of biological disorders or imbalances.

An additional embodiment of the present invention includes therapy and treatments mediated by NUCP gene delivery. Gene delivery can be to somatic or stem cells, and may be effected using viral (i.e., retrovirus, adeno-associated virus, etc.) or non-viral (i.e., cationic lipids, formulations using "naked" DNA, etc.) methods.

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequences of the NUCP polynucleotides, and the amino acid sequences encoded thereby.

5. DETAILED DESCRIPTION OF THE INVENTION

The NUCPs described for the first time herein are novel proteins that are expressed, inter alia, in gene trapped human cells, human lymph node or kidney cells, and/or ES cells. The NUCPs exert biological effect by regulating the efficiency of energy generation in the body with the result being that excess resources are converted to heat or are otherwise stored as fat, etc. Regulating the function of a NUCP product will effect NUCP-mediated processes with resulting effects on fat production and usage, superoxide generation and regulation, and all biological properties and functions that are tied to fatty acid metabolism. Because of these important roles, UCPs have been the focus of intense scientific scrutiny (see PCT Application No. PCT/EP98/02645, U.S. Pat. Nos. 5,853,975, 5,741,666 and 5,702,902 all of which are herein incorporated by reference in their entirety).

The present invention encompasses the use of the described NUCP nucleotides, NUCPs and NUCP peptides therefrom, as well as antibodies, preferably humanized monoclonal antibodies, or binding fragments, domains, or fusion proteins thereof, or antiidiotypic variants derived therefrom, that bind NUCP (which can, for example, also act as NUCP agonists or antagonists), other antagonists that inhibit binding activity or expression, or agonists that activate NUCP receptor activity or increase NUCP expression, in the diagnosis and/or treatment of disease.

In particular, the invention described in the subsections below encompasses NUCP polypeptides or peptides corresponding to functional domains of NUCPs, mutated, truncated or deleted NUCPs (e.g., NUCPs missing one or more functional domains or portions thereof), NUCP fusion proteins (e.g., where NUCP or a functional domain of NUCP is fused to an unrelated protein or peptide such as an immunoglobulin constant region, i.e., IgFc), nucleotide sequences encoding such products, and host cell expression systems that can produce such NUCP products.

The invention also encompasses antibodies and antiidiotypic antibodies (including Fab fragments), antagonists and agonists of the NUCP, as well as compounds or nucleotide constructs that inhibit expression of a NUCP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote expression of a NUCP (e.g., expression constructs in which a NUCP coding sequence is operatively associated with expression control elements such as promoters, promoter/enhancers, etc.). The invention also relates to host cells and animals genetically engineered to express a NUCP (or mutant variants thereof) or to inhibit or "knockout" expression of an animal homolog of a NUCP gene.

The NUCPs, NUCP peptides, and NUCP fusion proteins derived therefrom, NUCP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NUCPs or inappropriately expressed NUCPs for the diagnosis of biological disorders (high blood pressure, obesity, etc.) and disease. The NUCP products or peptides, NUCP fusion proteins, NUCP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can also be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NUCP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to an endogenous NUCP, but can also identify compounds that facilitate or inhibit NUCP-mediated uncoupling.

Of particular interest are genetically engineered nucleotide constructs, or expression vectors, that encode NUCP products and derivatives (NUCP peptides, fusions, etc). Nucleotide constructs encoding such NUCP products and derivatives can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NUCP product, NUCP peptide, or NUCP fusion protein to the body. Nucleotide constructs encoding functional NUCPs, mutant NUCPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NUCP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Therapeutic gene delivery of the described NUCP nucleotides can be effected by a variety of methods. For example,

methods of retroviral human gene therapy are described in, *inter alia*, U.S. Pat. Nos. 5,399,346 and 5,858,740; adenoviral vectors for gene therapy/delivery are described in U.S. Pat. No. 5,824,544; adeno-associated viral vectors are described in U.S. Pat. Nos. 5,843,742, 5,780,280, and 5,846,528; herpes virus vectors are described in U.S. Pat. No. 5,830,727, and other vectors and methods of nonvirally (e.g., polynucleotides that are not encapsulated by viral capsid protein, "naked" DNA, or DNA formulated in lipid or chemical complexes) introducing foreign genetic material of recombinant origin into a host mammalian, and preferably human, cell are described in U.S. Pat. Nos. 5,827,703 and 5,840,710 all of which are herein incorporated by reference in their entirety. When the above methods are applied to selectively express or inhibit the expression of a NUCP in tumor/diseased cells, the described methods and compositions can be used as chemotherapeutic agents for the treatment of cancer and other diseases and disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1. The NUCP Polynucleotides

The cDNA sequences (SEQ ID NOS:1 and 3) and deduced amino acid sequences (SEQ ID NOS:2 and 4) of the described NUCPs are presented in the Sequence Listing. The NUCP cDNA sequences were obtained from human lymph node, kidney, and fetal brain cDNA libraries (Edge Biosystems, Gaithersburg, Md.) using probes and/or primers generated from gene trapped sequence tags and a human homolog of the described NUCPs. RT-PCR analysis indicated that expression of the described NUCPs can be detected in, *inter alia*, human cerebellum, spinal cord, thymus, spleen, lymph node, bone marrow, trachea, lung, kidney, fetal liver, prostate, testis, thyroid, salivary gland, stomach, heart, uterus, and mammary gland, with particularly strong expression in kidney, adrenal gland, and skeletal muscle. The above expression studies were largely verified by Northern analysis that also detected particularly strong expression in human skeletal muscle, heart, adrenal gland, and kidney.

The NUCPs of the present invention include: (a) the human DNA sequences presented in the Sequence Listing and additionally contemplates any nucleotide sequence encoding a contiguous and functional NUCP open reading frame (ORF) that hybridizes to a complement of the DNA sequence presented in the Sequence Listing under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C., and washing in 0.1×SSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, e.g., washing in 0.2×SSC/0.1% SDS at 42° C. (Ausubel et al., 1989, *supra*), yet still encode a functionally equivalent NUCP product. Functional equivalents of a NUCP include naturally occurring NUCPs present in other species, and mutant NUCPs whether naturally occurring or engineered. The invention also includes degenerate variants of the disclosed sequences.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NUCP nucleotide

sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are particularly about 16 to about 100 bases long, about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the present Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, etc. Alternatively, the NUCP oligonucleotides can be used as hybridization probes for screening libraries or assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Chip applications can involve a series of the described NUCP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NUCP sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length may partially overlap each other and/or the NUCP sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described NUCP polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 18, and preferably about 25, nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences may begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37° C. (for 14-base oligos), 48° C. (for 17-base oligos), 55° C. (for 20-base oligos), and 60° C. (for 23-base oligos). These nucleic acid molecules may encode or act as NUCP gene antisense molecules, useful, for example, in NUCP gene regulation (for and/or as antisense primers in amplification reactions of NUCP gene nucleic acid sequences). With respect to NUCP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NUCP gene regulation.

Additionally, the antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and a hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 10 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NUCP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms, determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

Further, a NUCP gene homolog can be isolated from nucleic acid of the organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NUCP product disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue, such as choroid plexus, known or suspected to express a NUCP gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NUCP gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NUCP gene, such as, for example, brain tissue). A reverse transcription (RT) reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et al., 1989, *supra*.

A cDNA of a mutant NUCP gene may be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NUCP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NUCP allele to that of the normal NUCP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NUCP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry the mutant NUCP allele (e.g., a person manifesting a NUCP-associated phenotype such as, for example, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NUCP allele. The normal NUCP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NUCP allele in such libraries. Clones containing the mutant NUCP gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NUCP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal NUCP product as described below (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

Additionally, screening can be accomplished by screening with labeled NUCP fusion proteins, such as, for example, AP-NUCP or NUCP-AP fusion proteins. In cases where a NUCP mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), a polyclonal set of antibodies to NUCP are likely to cross-react with the mutant NUCP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known in the art.

The invention also encompasses nucleotide sequences that encode mutant NUCPs, peptide fragments of NUCPs,

truncated NUCPs, and NUCP fusion proteins. These include, but are not limited to nucleotide sequences encoding the mutant NUCPs described below; polypeptides or peptides corresponding to one or more domains of NUCP or portions of these domains; truncated NUCPs in which one or more of the domains is deleted, or a truncated nonfunctional NUCPs. Nucleotides encoding fusion proteins may include, but are not limited to, full length NUCP sequences, truncated NUCPs, or nucleotides encoding peptide fragments of a NUCP fused to an unrelated protein or peptide, such as for example, a NUCP domain fused to an Ig Fc domain which increases the stability and half life of the resulting fusion protein (e.g., NUCP-Ig) in the bloodstream; or an enzyme such as a fluorescent protein or a luminescent protein which can be used as a marker.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NUCP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NUCP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; (c) genetically engineered host cells that contain any of the foregoing NUCP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NUCP gene under the control of an exogenously introduced regulatory element (i.e., gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the cytomegalovirus hCMV immediate early gene, regulatable, viral (particularly retroviral LTR promoters) the early or late promoters of SV40 adenovirus, the lac system, the trp system, the tet system, the TAC system, the TRC system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

5.2. THE NUCPS and NUCP Polypeptides and Peptides Derived Therefrom

The NUCPs, NUCP polypeptides, NUCP peptide fragments, mutated, truncated, or deleted forms of a NUCP, and/or NUCP fusion proteins can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to a NUCP, as reagents in assays for screening for compounds that can be as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease.

The Sequence Listing discloses the amino acid sequences encoded by the described NUCP polynucleotides. The NUCP sequences both display initiator methionines that are present in a DNA sequence context consistent with a translation initiation site (Kozak sequence).

The NUCP sequences of the invention include the nucleotide and amino acid sequences presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NUCP homologues from other species are encompassed by the invention. In fact, any NUCP protein encoded by the NUCP nucleotide sequences described above are within the scope of the invention as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence

Listing. The degenerate nature of the genetic code is well known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, N.Y., herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NUCP encoded by the presently described nucleotide sequences, as judged by any of a number of criteria, including, but not limited to, the ability to partition into the mitochondria, or other cellular membrane structure, and effect uncoupling activity, change in cellular metabolism (e.g., ion flux, tyrosine phosphorylation, etc.), or change in phenotype when the NUCP equivalent is expressed at similar levels, or mutated, in an appropriate cell type (such as the amelioration, prevention or delay of a biochemical, biophysical, or overt phenotype). Functional equivalents of a NUCP include naturally occurring NUCPs present in other species and mutant NUCPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Pat. No. 5,837,458). The invention also includes degenerate nucleic acid variants and splice variant of the disclosed NUCP polynucleotide sequence.

Additionally contemplated are polynucleotides encoding NUCP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

Functionally equivalent NUCP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NUCP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

While random mutations can be made to NUCP encoding DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant NUCPs tested for activity, site-directed mutations of the NUCP coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant NUCPs with increased function, e.g., higher receptor binding affinity, decreased function, and/or increased physiological half-life, and increased signal transduction triggering. One starting point for such analysis is by aligning the disclosed human sequences with corresponding gene/protein sequences from, for example, other mammals

in order to identify amino acid sequence motifs that are conserved between different species. Non-conservative changes can be engineered at variable positions to alter function, signal transduction capability, or both. Alternatively, where alteration of function is desired, deletion or non-conservative alterations of the conserved regions (i.e., identical amino acids) can be engineered. For example, deletion or non-conservative alterations (substitutions or insertions) of the various conserved transmembrane domains.

Other mutations to a NUCP coding sequence can be made to generate NUCPs that are better suited for expression, scale up, etc. in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid in order to eliminate disulfide bridges; N-linked glycosylation sites can be altered or eliminated to achieve, for example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions of any one or more of the glycosylation recognition sequences which occur in an ECD (N-X-S or N-X-T), and/or an amino acid deletion at the second position of any one or more such recognition sequences in an ECD will prevent glycosylation of the NUCP at the modified tripeptide sequence. (See, e.g., Miyajima et al., 1986, EMBO J. 5(6):1193-1197).

Peptides corresponding to one or more domains of a NUCP, truncated or deleted NUCPs, as well as fusion proteins in which a full length NUCP, a NUCP peptide, or a truncated NUCP is fused to an unrelated protein, are also within the scope of the invention and can be designed on the basis of the presently disclosed NUCP gene nucleotide and NUCP amino acid sequences. Such fusion proteins include, but are not limited to, Ig Fc fusions which stabilize a NUCP protein, or NUCP peptides, and prolong half-life in vivo; or fusions to any amino acid sequence that allows the fusion protein to be anchored to the cell membrane; or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the NUCPs and NUCP peptides can be chemically synthesized (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W. H. Freeman & Co., N.Y.), large polypeptides derived from a full length NUCP can be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acids containing NUCP gene sequences and/or coding sequences. Such methods can be used to construct expression vectors containing the described NUCP nucleotide sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*. Alternatively, RNA corresponding to all or a portion of a transcript encoded by a NUCP gene sequence can be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M. J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems can be utilized to express the NUCP-encoding nucleotide sequences of the invention. Where a NUCP peptide or polypeptide is a soluble derivative (e.g., NUCP peptides corresponding to an ECD; truncated or deleted NUCP in which a TM and/or CD are deleted, etc.) the peptide can be recovered from the host

cell in cases where the NUCP peptide or polypeptide is not secreted, and from the culture media in cases where the NUCP peptide or polypeptide is secreted by the cells. However, such expression systems also encompass engineered host cells that express a NUCP, or a functional equivalent thereof, *in situ*, i.e., anchored in the cell membrane. Purification or enrichment of a NUCP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of a NUCP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that can be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NUCP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NUCP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing NUCP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NUCP nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NUCP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NUCP, or for raising antibodies to a NUCP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NUCP coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polydrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The NUCP gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the NUCP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded

recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NUCP gene nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing NUCP in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of NUCP transcripts. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NUCP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed (for example an independent ribosome entry site, or IRES, site). However, in cases where only a portion of a NUCP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can have a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the presently described NUCPs can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells can be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to

form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express a NUCP. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NUCP product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

NUCP products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate NUCP transgenic animals.

Any technique known in the art may be used to introduce a NUCP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry a NUCP transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al., 1992, Proc. Natl.

Acad. Sci. USA 89:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the NUCP transgene be integrated into the chromosomal site of the endogenous NUCP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NUCP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NUCP gene (i.e., "knockout" animals).

The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous NUCP gene in only that cell type, by following, for example, the teaching of Gu et al., 1994, Science, 265:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NUCP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR. Samples of NUCP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the NUCP transgene product.

5.3. Antibodies to NUCPS

Antibodies that specifically recognize one or more epitopes of a NUCP, or epitopes of conserved variants of a NUCP, or peptide fragments of a NUCP are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention can be used, for example, in the detection of a NUCP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of a NUCP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described below, for the evaluation of the effect of test compounds on expression and/or activity of a NUCP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NUCP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for inhibiting abnormally high NUCP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with a NUCP, a NUCP peptide (e.g., one corresponding to a functional domain of a NUCP), truncated NUCP polypeptides (a NUCP in which one or more domains have been deleted), functional equivalents of the NUCP or mutants of the NUCP. Such host animals may include but are not limited to rabbits, mice,

goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, key-hole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against NUCP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes can be generated using known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NUCP can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" a given NUCP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies that bind to a NUCP domain and competitively inhibit the binding of a NUCP to its cognate ligand, chaperonin, or accessory molecule(s) can be used to

generate anti-idiotypes that "mimic" the NUCP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NUCP-mediated process or pathway.

5.4. Diagnosis of Abnormalities Related to a NUCP

A variety of methods can be employed for the diagnostic and prognostic evaluation of disorders related to NUCP function, and for the identification of subjects having a predisposition to such disorders.

Such methods may, for example, utilize reagents such as the NUCP nucleotide sequences described above and the NUCP antibodies described above. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of NUCP gene mutations, or the detection of either over- or under-expression of NUCP mRNA relative to a given phenotype; (2) the detection of either an over- or an under-abundance of NUCP gene product relative to a given phenotype; and (3) the detection of perturbations or abnormalities in any metabolic, physiologic, or catabolic pathway mediated by NUCP.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific NUCP nucleotide sequence or NUCP antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting, for example, body weight disorder abnormalities.

For the detection of NUCP mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of NUCP gene expression or NUCP gene products, any cell type or tissue in which the NUCP gene is expressed, such as, for example, kidney cells, may be utilized.

Nucleic acid-based detection techniques are described, below, in Section 5.4.1. Peptide detection techniques are described, below, in Section 5.4.2.

5.4.1. Detection of NUCP Sequences

Mutations within a NUCP nucleotide sequence can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and can be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving NUCP gene structure, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

Such diagnostic methods for the detection of NUCP gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described above, under conditions favorable for the specific annealing of these reagents to their complementary sequences within a NUCP gene. Preferably, the lengths of these nucleic acid reagents are at least about 15 to about 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:NUCP molecule hybrid.

The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described above are easily removed. Detection of the remaining annealed, labeled NUCP nucleic acid reagents is accomplished using standard techniques well known to those in the art. The NUCP encoding nucleotide sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal NUCP gene sequence in order to determine whether a NUCP gene mutation is present.

Alternative diagnostic methods for the detection of NUCP gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K. B., 1987, U.S. Pat. No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of a NUCP gene in order to determine whether a NUCP gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals carrying NUCP gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

Additionally, improved methods for analyzing DNA polymorphisms which can be utilized for the identification of NUCP gene mutations have been described which capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217, which is incorporated herein by reference in its entirety) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)_n-(dG-dT)_n short tandem repeats. The average separation of (dC-dA)_n-(dG-dT)_n blocks is estimated to be 30,000-60,000 bp. Markers which are so closely spaced exhibit a high frequency co-inheritance, and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the NUCP gene, and the diagnosis of diseases and disorders related to NUCP mutations.

Also, Caskey et al. (U.S. Pat. No. 5,364,759, which is incorporated herein by reference in its entirety) describe a DNA profiling assay for detecting short tri and tetra nucleotide repeat sequences. The process includes extracting the DNA of interest, such as the NUCP gene, amplifying the extracted DNA, and labeling the repeat sequences to form a genotypic map of the individual's DNA.

The level of NUCP gene expression can also be assayed by detecting and measuring NUCP transcription. For example, RNA from a cell type or tissue known, or suspected to express the NUCP gene, such as kidney, may be isolated and tested utilizing hybridization or PCR techniques such as those described above. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the NUCP gene. Such analyses may reveal both quantitative and qualitative aspects

of the expression pattern of the NUCP gene, including activation or inactivation of NUCP gene expression.

In one embodiment of such a detection scheme, cDNAs are synthesized from the RNAs of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the NUCP nucleic acid reagents described above. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining, by utilizing any other suitable nucleic acid staining method, or by sequencing.

Additionally, it is possible to perform such NUCP gene expression assays "in situ", i.e., directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described in Section 5.1 may be used as probes and/or primers for such in situ procedures (See, for example, Nuovo, G. J., 1992, "PCR In Situ Hybridization: Protocols And Applications", Raven Press, N.Y.).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern analysis can be performed to determine the level of mRNA expression of the NUCP gene.

5.4.2. Detection of NUCP Products

Antibodies directed against wild type or mutant NUCPs, or conserved variants or peptide fragments thereof, as discussed above, can also be used as diagnostics and prognostics, as described herein. Such diagnostic methods, may be used to detect abnormalities in the level of NUCP gene expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of the NUCP (besides mitochondria), and may be performed in vivo or in vitro, such as, for example, on biopsy tissue.

For example, antibodies directed to one or more epitopes of NUCP can be used in vivo to detect the pattern and level of expression of NUCP in the body. Such antibodies can be labeled, e.g., with a radio-opaque or other appropriate compound and injected into a subject in order to visualize binding to the NUCP expressed in the body using methods such as X-rays, CAT-scans, or MRI. Labeled antibody fragments, e.g., the Fab or single chain antibody comprising the smallest portion of the antigen binding region, are preferred for this purpose to promote crossing the blood-brain barrier and permit labeling of NUCP expressed in the brain.

Additionally, any NUCP fusion protein or NUCP conjugated protein whose presence can be detected, can be administered. For example, NUCP fusion or conjugated proteins labeled with a radio-opaque or other appropriate compound can be administered and visualized in vivo, as discussed, above for labeled antibodies. Further such NUCP fusion proteins (such as AP-NUCP or NUCP-AP) can be utilized for in vitro diagnostic procedures.

Alternatively, immunoassays or fusion protein detection assays, as described above, can be utilized on biopsy and autopsy samples in vitro to permit assessment of the expres-

sion pattern of the NUCP. Such assays are not confined to the use of antibodies that define a NUCP domain, but can include the use of antibodies directed to epitopes of any domain of a NUCP. The use of each or all of these labeled antibodies will yield useful information regarding translation and intracellular transport of the NUCP to the cell surface and can identify defects in processing.

The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the NUCP gene, such as, for example, epithelial cells, kidney cells, adipose tissue, brain cells, etc. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the NUCP gene.

For example, antibodies, or fragments of antibodies, such as those described above useful in the present invention may be used to quantitatively or qualitatively detect the presence of a NUCP, or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below, this Section) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred if such NUCP products can be found, at least transiently, on the cell surface.

The antibodies (or fragments thereof) or NUCP fusion or conjugated proteins useful in the present invention may additionally be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for in situ detection of NUCP gene products or conserved variants or peptide fragments thereof, or to assay NUCP binding (in the case of labeled NUCP-fusion protein).

In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody or fusion protein of the present invention. The antibody (or fragment) or fusion protein is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the NUCP product, or conserved variants or peptide fragments, or NUCP binding, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Immunoassays and non-immunoassays for a NUCP, or conserved variants or peptide fragments thereof, will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying NUCP products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art. Alternatively, the labeled antibody can be directed against an antigenic tag that has been directly or indirectly attached to a NUCP.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled NUCP antibody or NUCP ligand/accessory molecule fusion protein. The solid phase support may then be washed with the buffer a second time to remove unbound antibody or fusion protein. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of NUCP antibody or NUCP ligand fusion protein may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

With respect to antibodies, one of the ways in which the NUCP antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, Md.); Voller, A. et al., 1978, J. Clin. Pathol. 31:507-520; Butler, J. E., 1981, Meth. Enzymol. 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, Fla.; Ishikawa, E. et al., (eds.), 1981, Enzyme Immunoassay, Kaku Shoin, Tokyo). The enzyme that is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by calorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect NUCP through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles

of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, thermotropic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

5.5. Screening Assays for Compounds that Modulate NUCP Expression or Activity

The following assays are designed to identify compounds that interact with (e.g., bind to) a NUCP, compounds that interfere with the interaction of a NUCP with any ligand or accessory molecules, compounds that modulate NUCP gene expression (i.e., modulate the level of NUCP activity by regulating gene expression) or otherwise modulate the levels of a NUCP in the body. Assays may additionally be utilized which identify compounds that bind to NUCP gene regulatory sequences (e.g., promoter sequences) and, consequently, may modulate NUCP gene expression. See e.g., Platt, K. A., 1994, *J. Biol. Chem.* 269:28558-28562, which is incorporated herein by reference in its entirety.

The compounds which can be screened in accordance with the invention include but are not limited to peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that bind to NUCP and either mimic the activity of the natural product (i.e., agonists) or inhibit the activity of the natural ligand/accessory molecule (i.e., antagonists); as well as peptides, antibodies or fragments thereof, and other organic compounds that mimic the NUCP (or a portion thereof) and bind to and "inactivate" or "neutralize" the NUCP ligand/accessory protein.

Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam, K. S. et al., 1991, *Nature* 354:82-84; Houghten, R. et al., 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L-

configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang, Z. et al., 1993, *Cell* 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab)₂ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Other compounds that can be screened in accordance with the invention include but are not limited to small organic molecules that are able to cross the blood-brain barrier, gain entry into an appropriate cell (e.g., in the choroid plexus, pituitary, the hypothalamus, etc.) and affect the expression of a NUCP gene or some other gene involved in a NUCP mediated pathway (e.g., by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect or substitute for the activity of the NUCP or the activity of some other intracellular factor involved in a NUCP-mediated catabolic, or metabolic pathway.

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate NUCP expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be ligand binding sites. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site (or binding site), either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a

search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential NUCP modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved specificity or activity.

Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites (or binding sites) of NUCP, and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA programs (Polygen Corporation, Waltham, Mass.). CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotinenen, et al., 1988, *Acta Pharmaceutical Fennica* 97:159-166; Ripka, *New Scientist* 54-57 (Jun. 16, 1988); McKinaly and Rossmann, 1989, *Annu. Rev. Pharmacol. Toxicol.* 29:111-122; Perry and Davies, *OSAR: Quantitative Structure-Activity Relationships in Drug Design* pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 *Proc. R. Soc. Lond.* 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989, *J. Am. Chem. Soc.* 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, Calif.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Cell-based systems can also be used to identify compounds that bind (or mimic) NUCP as well as assess the altered activity associated with such binding in living cells. One tool of particular interest for such assays is green fluorescent protein which is described, inter alia, in U.S. Pat. No. 5,625,048, herein incorporated by reference. Cells that may be used in such cellular assays include, but are not limited to, leukocytes, or cell lines derived from leukocytes, lymphocytes, stem cells, including embryonic stem cells,

and the like. In addition, expression host cells (e.g., B95 cells, COS cells, CHO cells, OMK cells, fibroblasts, Sf9 cells) genetically engineered to express a functional NUCP of interest and to respond to activation by the test, or natural, ligand, as measured by a chemical or phenotypic change, or induction of another host cell gene, can be used as an end point in the assay.

Compounds identified via assays such as those described herein may be useful, for example, in elucidating the biological function of NUCP. Such compounds can be administered to a patient at therapeutically effective doses to treat any of a variety of physiological or mental disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in any amelioration, impediment, prevention, or alteration of any biological symptom.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, intracranial, intrathecal, or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions,

syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

5.5.1. In Vitro Screening Assays for Compounds that Bind to at NUCP

In vitro systems may be designed to identify compounds capable of interacting with (e.g., binding to) or mimicking a NUCP. The compounds identified can be useful, for example, in modulating the activity of wild type and/or

mutant NUCP; can be useful in elaborating the biological function of NUCP; can be utilized in screens for identifying compounds that disrupt normal NUCP interactions; or may themselves disrupt or activate such interactions.

The principle of the assays used to identify compounds that bind to a NUCP, or NUCP ligands/accessory molecules, involves preparing a reaction mixture of NUCP and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The NUCP species used can vary depending upon the goal of the screening assay. For example, where agonists of a natural NUCP accessory molecule or ligand are desired, a full length NUCP, or a soluble truncated NUCP, a NUCP peptide, or NUCP fusion protein containing one or more NUCP domains fused to a protein or polypeptide that affords advantages in the assay system (e.g., labeling, isolation of the resulting complex, etc.) can be utilized. Where compounds that directly interact with a NUCP are sought, peptides corresponding to NUCP and fusion proteins containing a NUCP, or a portion thereof, can be used.

The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring a NUCP, NUCP polypeptide, NUCP peptide, or fusion protein thereof, or the test substance onto a solid phase and detecting NUCP/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the NUCP reactant may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for a NUCP, NUCP polypeptide, peptide or fusion protein, or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Alternatively, cell-based assays can be used to identify compounds that interact with a NUCP. To this end, cell lines

that express a NUCP, or cell lines (e.g., COS cells, CHO cells, fibroblasts, etc.) that have been genetically engineered to express a NUCP or a NUCP ligand/accessory molecule (e.g., by transfection or transduction of NUCP DNA, etc.) can be used. Interaction of the test compound with, for example, NUCP ligand expressed by the host cell can be determined by comparison or competition with native NUCP.

5.5.2. Assays for Compounds that Interfere with NUCP Receptor/Intracellular or NUCP/Transmembrane Macromolecule Interaction

Macromolecules that interact with a NUCP are referred to, for purposes of this discussion, as "binding partners". These binding partners are likely to be involved in NUCP mediated biological pathways. Therefore, it is desirable to identify compounds that interfere with or disrupt the interaction of such binding partners which may be useful in regulating or augmenting NUCP activity in the body and/or controlling disorders associated with NUCP activity (or a deficiency thereof).

The basic principle of the assay systems used to identify compounds that interfere with the interaction between NUCP, or NUCP polypeptides, peptides or fusion proteins as described above (collectively, the NUCP moiety), and its binding partner or partners involves preparing a reaction mixture containing the NUCP moiety and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the NUCP moiety and its binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the NUCP moiety and the binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the NUCP moiety and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal NUCP may also be compared to complex formation within reaction mixtures containing the test compound and a mutant NUCP. This comparison may be important in those cases wherein it is desirable to identify compounds that specifically disrupt interactions of mutant, or mutated, NUCPs but not normal NUCPs.

The assay for compounds that interfere with the interaction of the NUCP moiety and its binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the NUCP moiety or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to, or simultaneously with, the NUCP moiety and interactive binding partner. Alternatively, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the NUCP moiety or an interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the NUCP moiety or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the NUCP moiety and an interactive binding partner is prepared in which either the NUCP moiety or its binding partners is labeled, but the signal generated by the label is quenched due to formation of the complex (see, e.g., U.S. Pat. No. 4,190,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt NUCP/intracellular binding partner interaction can be identified.

In a particular embodiment, a NUCP fusion can be prepared for immobilization. For example, NUCP or a peptide fragment can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art and/or described above. This antibody can be labeled with the radioactive isotope ^{125}I , for example, by methods routinely practiced in the art. In a heterogeneous assay, e.g., the GST-NUCP fusion protein can be anchored to glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test compound in a manner that allows

interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the NUCP moiety and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-NUCP moiety fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the NUCP moiety/binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domain(s) of the NUCP moiety and/or the interactive or binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensatory mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described above, and allowed to

interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a relatively short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the intracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, the NUCP moiety can be anchored to a solid material as described, above, by making a GST-NUCP moiety fusion protein and allowing it to bind to glutathione agarose beads. The interactive binding partner can be labeled with a radioactive isotope, such as ^{35}S , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-NUCP moiety fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the intracellular binding partner binding domain, can be eluted, purified, and analyzed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. All patents, patent applications, and publications cited herein are hereby incorporated by reference.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1

<211> LENGTH: 876

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

```

atgtcagccc tcaactggaa gccgtttgtg tacggggggc tggcctccat c actgctgag      60
tgcggtacat ttccaattga ttaaccaag acacggctcc agattcaagg c cagacgaat      120
gatgcaaaat ttaaggaaat tagataccga ggaatgttc acgcattagt g aggataggc      180
agagaagaag ggcgtgaaagc actctactcg gggattgccc ccgcgatgtt a cgccaggca      240
tcttatggca ccatcaagat aggcacttac cagagcttga agcgactatt c attgaacgc      300
ccagaagatg aaactctacc gataaatgtg atatgtggaa ttctgtcttg a gtcatatct      360
tcaaccattg ctaatccaac tgatgttttg aaaattcgga tgcaagcgca a agcaacacc      420
attcaaggag gaatgatagg caacttcacg aacatttacc agcaagaggg g acaagagga      480
ctgtggaagg gtgtgtccct tactgcgcag agggctgcta ttgttgttgg t gtggagctg      540
ccggtctatg acatcaccaa gaagcatctt attctctcag gcctgatggg a gacactgtg      600

```

-continued

tatacccact tctctcaag cttcacctgt ggtctggcag gggccctggc c tcaaaccct 660
 gttgatgttg tgaggacacg tatgatgaat cagagagtgc ttcgagatgg c agatgttct 720
 ggctacacag gaaccctgga ttgcttgta cagacatgga agaataagg g ttttttgc 780
 ctctataaag gcttttggcc aaattggtg agacttggtc cttggaatat c attttctt 840
 gtgacatacg agcagttgaa gaaattggat ttgtga 876

<210> SEQ ID NO 2
 <211> LENGTH: 291
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Ser Ala Leu Asn Trp Lys Pro Phe Val T yr Gly Gly Leu Ala Ser
 1 5 10 15
 Ile Thr Ala Glu Cys Gly Thr Phe Pro Ile A sp Leu Thr Lys Thr Arg
 20 25 30
 Leu Gln Ile Gln Gly Gln Thr Asn Asp Ala L ys Phe Lys Glu Ile Arg
 35 40 45
 Tyr Arg Gly Met Leu His Ala Leu Val Arg I le Gly Arg Glu Glu Gly
 50 55 60
 Leu Lys Ala Leu Tyr Ser Gly Ile Ala Pro A la Met Leu Arg Gln Ala
 65 70 75 80
 Ser Tyr Gly Thr Ile Lys Ile Gly Thr Tyr G ln Ser Leu Lys Arg Leu
 85 90 95
 Phe Ile Glu Arg Pro Glu Asp Glu Thr Leu P ro Ile Asn Val Ile Cys
 100 105 110
 Gly Ile Leu Ser Gly Val Ile Ser Ser Thr I le Ala Asn Pro Thr Asp
 115 120 125
 Val Leu Lys Ile Arg Met Gln Ala Gln Ser A sn Thr Ile Gln Gly Gly
 130 135 140
 Met Ile Gly Asn Phe Met Asn Ile Tyr Gln G ln Glu Gly Thr Arg Gly
 145 150 155 160
 Leu Trp Lys Gly Val Ser Leu Thr Ala Gln A rg Ala Ala Ile Val Val
 165 170 175
 Gly Val Glu Leu Pro Val Tyr Asp Ile Thr L ys Lys His Leu Ile Leu
 180 185 190
 Ser Gly Leu Met Gly Asp Thr Val Tyr Thr H is Phe Leu Ser Ser Phe
 195 200 205
 Thr Cys Gly Leu Ala Gly Ala Leu Ala Ser A sn Pro Val Asp Val Val
 210 215 220
 Arg Thr Arg Met Met Asn Gln Arg Val Leu A rg Asp Gly Arg Cys Ser
 225 230 235 240
 Gly Tyr Thr Gly Thr Leu Asp Cys Leu Leu G ln Thr Trp Lys Asn Glu
 245 250 255
 Gly Phe Phe Ala Leu Tyr Lys Gly Phe Trp P ro Asn Trp Leu Arg Leu
 260 265 270
 Gly Pro Trp Asn Ile Ile Phe Phe Val Thr T yr Glu Gln Leu Lys Lys
 275 280 285
 Leu Asp Leu
 290

<210> SEQ ID NO 3
 <211> LENGTH: 882

-continued

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

```

atgtcagccc tcaactggaa gccgtttgtg tacggggggg tggcctccat c actgctgag      60
tgcggtacat ttccaattga ttaaccaag acacggctcc agattcaagg c cagacgaat      120
gatgcaaaat ttaaggaaat tagataccga ggaatgttg acgcattagt g aggataggc      180
agagaagaag ggctgaaagc actctactcg gggattgccc ccgcgatgtt a cgccaggca      240
tcctatggca ccatcaagat aggcacttac cagagcttga agcgactatt c attgaacgc      300
ccagaagatg aaactctacc gataaatgtg atatgtggaa ttctgtctgg a gtcatatct      360
tcaaccattg ctaatccaac tgatgttttg aaaattcgga tgcaagcgca a agcaacacc      420
attcaaggag gaatgatagg caacttcacg aacatttacc agcaagaggg g acaagagga      480
ctgtggaagg gtgtgtccct tactgcgcag agggctgcta ttgttgttg t gtggagctg      540
ccggtctatg acatcaccaa gaagcatctt attctctcag gcctgatggg a gacactgtg      600
tatacccaat tcctctcaag cttcacctgt ggtctggcag gggccctggc c tcaaacctt      660
gttgatgttg tgaggacacg tatgatgaat cagagagtgc ttcgagatgg c agatgttct      720
ggctacacag gaaccttgga ttgcttgta cagcttacag tgctggaaag t tttccacc      780
acagcaaaag cacaagaagct tatcagcgta gatgccatct cagaagaggc t gataccagg      840
ggattttacat atctcagctg tgatctttct gtcctcaagct ga                        882

```

<210> SEQ ID NO 4

<211> LENGTH: 293

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

```

Met Ser Ala Leu Asn Trp Lys Pro Phe Val T yr Gly Gly Leu Ala Ser
 1             5             10             15
Ile Thr Ala Glu Cys Gly Thr Phe Pro Ile A sp Leu Thr Lys Thr Arg
      20             25             30
Leu Gln Ile Gln Gly Gln Thr Asn Asp Ala L ys Phe Lys Glu Ile Arg
      35             40             45
Tyr Arg Gly Met Leu His Ala Leu Val Arg I le Gly Arg Glu Glu Gly
      50             55             60
Leu Lys Ala Leu Tyr Ser Gly Ile Ala Pro A la Met Leu Arg Gln Ala
      65             70             75             80
Ser Tyr Gly Thr Ile Lys Ile Gly Thr Tyr G ln Ser Leu Lys Arg Leu
      85             90             95
Phe Ile Glu Arg Pro Glu Asp Glu Thr Leu P ro Ile Asn Val Ile Cys
      100            105            110
Gly Ile Leu Ser Gly Val Ile Ser Ser Thr I le Ala Asn Pro Thr Asp
      115            120            125
Val Leu Lys Ile Arg Met Gln Ala Gln Ser A sn Thr Ile Gln Gly Gly
      130            135            140
Met Ile Gly Asn Phe Met Asn Ile Tyr Gln G ln Glu Gly Thr Arg Gly
      145            150            155            160
Leu Trp Lys Gly Val Ser Leu Thr Ala Gln A rg Ala Ala Ile Val Val
      165            170            175
Gly Val Glu Leu Pro Val Tyr Asp Ile Thr L ys Lys His Leu Ile Leu
      180            185            190

```

-continued

Ser Gly Leu Met Gly Asp Thr Val Tyr Thr H is Phe Leu Ser Ser Phe
 195 200 205

Thr Cys Gly Leu Ala Gly Ala Leu Ala Ser A sn Pro Val Asp Val Val
 210 215 220

Arg Thr Arg Met Met Asn Gln Arg Val Leu A rg Asp Gly Arg Cys Ser
 225 230 235 240

Gly Tyr Thr Gly Thr Leu Asp Cys Leu Leu G ln Leu Thr Val Leu Glu
 245 250 255

Ser Phe Ser Thr Thr Ala Lys Pro Gln Lys L eu Ile Ser Val Asp Ala
 260 265 270

Ile Ser Glu Glu Ala Asp Thr Arg Gly Phe T hr Tyr Leu Ser Cys Asp
 275 280 285

Leu Ser Ala Pro Ser
 290

What is claimed is:

1. An isolated nucleic acid molecule comprising at least 24 contiguous bases of a human nucleotide sequence from the uncoupling protein polynucleotide sequence described in SEQ ID NO: 1.
2. An isolated nucleic acid molecule comprising a human nucleotide sequence that:
 - (a) encodes the amino acid sequence shown in SEQ ID NO: 2; and
 - (b) hybridizes under highly stringent conditions to the nucleotide sequence of SEQ ID NO: 1 or the complement thereof.
3. An isolated nucleic acid molecule comprising a human nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2.
4. An isolated nucleic acid molecule comprising the human nucleotide sequence of the uncoupling protein polynucleotide sequence described in SEQ ID NO: 3.
5. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 4.

* * * * *

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ BLACK BORDERS

☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

☐ FADED TEXT OR DRAWING

☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING

☐ SKEWED/SLANTED IMAGES

☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS

☐ GRAY SCALE DOCUMENTS

☐ LINES OR MARKS ON ORIGINAL DOCUMENT

☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.